ISOLATION OF NON-FERMENTING GRAM NEGATIVE BACILLI FROM INTENSIVE CARE UNIT PATIENTS WITH SPECIAL REFERENCE TO METALLO BETALACTAMASE PRODUCTION IN PSEUDOMONAS

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CERTIFICATE

This is to certify that this dissertation entitled "**ISOLATION OF NON-FERMENTING GRAM NEGATIVE BACILLI FROM INTENSIVE CARE UNIT PATIENTS WITH SPECIAL REFERENCE TO METALLOBETA LACTAMASE PRODUCTION IN PSEUDOMONAS"** is the bonafide original work done by **Dr.S.MATHAVI,** Post graduate in Microbiology, under my overall supervision and guidance in the Department of Microbiology, Stanley Medical College, Chennai, in partial fulfillment of the regulations of The Tamil Nadu Dr.M.G.R. Medical University for the award of **M.D.Degree in Microbiology (Branch IV).**

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DECLARATION

I solemnly declare that this dissertation "**ISOLATION OF NON-FERMENTING GRAM NEGATIVE BACILLI FROM INTENSIVE CARE UNIT PATIENTS WITH SPECIAL REFERENCE TO METALLOBETA LACTAMASE PRODUCTION IN PSEUDOMONAS IN GOVT. STANLEY HOSPITAL, CHENNAI"** is the bonafide work done by me at the Department of Microbiology, Govt, Stanley Medical College and Hospital, Chennai, under the guidance and supervision of Prof. **Dr.P.R.THENMOZHI VALLI,M.D.,** Professor & H.O.D, Department of Microbiology, Govt. Stanley Medical College, Chennai – 600 001.

This dissertation is submitted to The Tamil Nadu Dr.M.G.R. Medical University, Chennai in partial fulfillment of the University regulations for the award of degree of M.D. Branch IV Microbiology examinations to be held in March 2008.

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CONTENTS

Introduction

INTRODUCTION

The term “non-fermenters”(NFs) refers to a group of aerobic, nonspore forming Gram negative bacilli (GNB) that are either incapable of utilizing carbohydrate as a source of energy or degrade them via “oxidative” rather than fermentative pathways.[55]

In addition, they possess cytochrome oxidase activity and fail to grow or grow poorly on MacConkey agar.[14] Although frequently considered to be commensals or contaminants, the pathogenic potential of non-fermenters has been established beyond doubt by their recurrent isolation from clinical materials and their association with human disease.[3]

The critically ill patients receiving life saving therapy in Intensive care unit (ICU) often requires broad spectrum antibiotics, insertion of various indwelling devices, nutritional support and immuno-suppressive therapy.[9] These factors along with underlying debility and immunosuppression account for the fact that though only 5-10% of all hospital beds are in ICU, these patients happen to be victims of over 20% of all hospital acquired infections. It is in this modern context of intensive treatment that the importance of non-fermenters as a nosocomial pathogen has increased.[21]

Pseudomonas aeruginosa is one of the main pathogenic agent in this group and ranks second among all pathogens evidenced in prevalence of infections in Intensive care study.[27] Ventilator associated pneumonia caused by this microorganism remains a severe and dreaded complication.[20]

Acinetobacter is now been increasingly recognized as cause of respiratory tract infection in ICU patients with *Acinetobacter baumanii* is the commonest species.[29] Other non-fermenter which is gaining importance in

ICU patients is *Stenotrophomonas maltophilia*. This is associated with increased length of ICU stay, duration of mechanical ventilator support and mortality among patients with ventilator associated pneumonia.[49]

P.aeruginosa is a classical opportunistic pathogen with innate resistance to many antibiotics.[6] An alarming increase in the resistance to various antimicrobial agents have been reported from India and abroad. They exhibit alarming levels of resistance to even most recent antibiotics like third generation cephalosporins, antipseudomonal penicillins (Piperacillin, Ticarcillin), aminoglycosides and fluoroquinolones. [13,19]

Resistance to these agents can arise by various mechanisms including mutational depression of AmpC chromosomal beta-lactamases, acquisition of secondary plasmids or transposon mediated beta-lactamases, reduced permeability or multidrug efflux[25] or in case of imipenem via, loss of D2 porin.[11]

The introduction of Carbapenems (meropenem, imipenem) into clinical practise represented a greater advance for treatment of serious bacterial infections caused by beta –lactam resistant bacteria. However, carbapenem resistance has been frequently observed in non-fermenting bacilli like *P.aeruginosa* and Acinetobacter species. The common form of resistance is mediated by lack of drug penetration and/or carbapenem-hydrolysing beta-lactamases.[25] In this study importance in isolation of non-fermenting gram negative bacilli(NFGNB) and establish their pathogenesis by repeat isolation, to speciate the isolates and study their susceptibility patterns and to determine the presence of metallo-betalactamase (MBL) producing Pseudomonas from patients admitted to ICU are discussed.

Review of Literature

REVIEW OF LITERATURE

The group of non-fermenters include several genera and species of bacteria with special growth requirements and the dividing line between what is a “non-fermenter” and what may otherwise be designated as “fastidious” non-glucose fermenting GNB is based more on convention than on well-defined genetic or phenotypic characteristics.[30] As these organisms are inhabitants of environment and they occurred on body surface and mucous membranes, until recently ,a number of non-fermenters were looked upon as non-pathogenic commensals of little clinical significance. But recently, there has been a tremendous interest in these organisms as they can cause disease by colonizing and subsequently infecting immunocompromised individuals. The pathogenic potential of non-fermenters is established beyond doubt by their repeated isolation from clinical samples and their association with disease.[3]

The range of infections caused by these organisms vary from cutaneous infections to primary bacteremias. Urinary tract infections(UTI), post-operative wound infections, pneumonias and primary bacteremias make upto 80-90% of reported infections. The commonly acquired non-fermenters in community based as well as hospitalized patients are Pseudomonads species, Acinetobacter, Alcaligenes and *Stenotrophomonas maltophilia*.[55]

MEDICALLY IMPORTANT NON-FERMENTERS

Motile with polar flagella, Oxidase Positive

Family Pseudomonadaceae

(rRNA group 1)

Genus Pseudomonas (except P.luteola, P.oryzihabitans)

Family Burkholderiaceae

(rRNA group 2)

Genus Burkholderia (except B.cepacia complex, B.gladioli)

Genus Cupriavidus

Genus Lautropia

Genus Pandoraea

Genus Ralstonia

Family Comamonadaceae

(rRNAgroup 3)

Genus Comamonas

Genus Acidovorax

Genus Delftia

Family Caulobacteraceae

(rRNAgroup 4)

Genus Brevundimonas

Family Xanthomonadaceae

(rRNA group5)

Genus Stenotrophomonas (except S.maltophilia)

Family Sphingomonadaceae

Genus Sphingomonas

Family Oceanospirillaceae

Genus Balneatrix

Family Alteromonadaceae

Genus Alishewanella

Genus Shewanella

Family Oxalobacteraceae

Genus Herbaspirillum

Genus Massilia

Family Methylobacteriaceae

Genus Methylobacterium

Genus Roseomomas

Motile with peritrichous flagella

Family Alcaligenaceae

Genus Achromobacter

Genus Alcaligenes

Genus Bordetella (B.avium, B.bronchiseptica, B.hinzii)

Genus Kerstersia

Genus Oligella (O.ureolytica)

Family Rhizobiaceae

Genus Rhizobium

Family Brucellaceae

Genus Ochrobactrum

Family Halomonadaceae

Genus Halomonas

Nonmotile, Oxidase Positive

Family Flavobacteriaceae

Genus Flavobacterium

Genus Bergeyella

Genus Chryseobacterium

Genus Empedobacter

Genus Myroides

Genus Weeksella

Family Sphingobacteriaceae

Genus Sphingobacterium

Genus Pedobacter

Family Moraxellaceae

Genus Moraxella

Genus Psychrobacter

Family Neisseriaceae

Genus Neisseria

Family Alcaligenaceae

Genus Oligella (O.urethralis)

Family Rhodobacteraceae

Genus Paracoccus

Nonmotile, Oxidase – Negative

Family Moraxellaceae

Genus Acinetobacter

Family Alcaligenaceae

Genus Bordetella(B.pertussis,B.parapertussis,B.trematum)

Organism whose taxonomic position is uncertain

CDC group NO-1

CDC group EO-5

INITIAL CLUES THAT AN UNKNOWN ISOLATE IS A NON-FERMENTER

- ❖ Lack of evidence for glucose fermentation.
- ❖ Positive cytochrome oxidase test.
- ❖ Failure to grow on MacConkey agar.

Lack Of Evidence For Glucose Fermentation

Acid produced by NFs are considerably weaker than mixed acids derived from fermentative bacteria, thus the pH in fermentation test media in which a NF is growing may not drop sufficiently to convert the pH indicator. The initial clue that an unknown organism is a NF is usually the lack of acid production in either Triple sugar iron(TSI) or Kligler iron agar(KIA) media, manifested as an alkaline slant and an alkaline deep.[31]

Positive Cytochrome Oxidase Reaction

Any colony of a GNB growing on blood agar or any other primary isolation media that is cytochrome oxidase positive can be suspected of belonging to NF group. To test the oxidase activity of NFs, CDC recommends using a 0.5% aqueous solution of tetramethyl p-phenylene diamine dihydrochloride. A few drops of reagent can be used to flood the surface of agar medium on which bacterial colonies are growing. The development of blue color within a few seconds indicate a positive test. Negative test can be confirmed using the more sensitive Kovac's method, in which loopful of organisms is mixed with few drops of 1% tetramethyl p-phenylene diamine dihydrochloride reagent on a piece of filter paper. The development of dark blue color in 10 seconds indicates a positive test.[31]

Failure To Grow On MacConkey Medium

GNB that grows on blood agar but poorly or not at all on Macconkey agar should be suspected of belonging to NF group.[31]

TESTS USED FOR IDENTIFICATION OF NON-FERMENTERS[31]

Utilisation Of Glucose

Hugh-Leifson Oxidative-Fermentative(OF) medium is recommended to detect the metabolic properties of NFGNB. Two tubes of each carbohydrate medium are required for the test. The medium in one tube is exposed to air; the other is overlaid with sterile mineral oil or melted paraffin. Oxidative organisms produce acid only in the open tube exposed to atmospheric oxygen; fermenting organisms produce acid in both the tubes; and non-

saccharolytic organisms are inert in both the tubes, which remains at an alkaline pH after incubation.

Motility

The hanging drop preparation may be more accurate in detecting motility of NFGNB. A loopful of 6 to 24 hr, actively growing broth culture that has been incubated at 37^{o}C is placed in the center of No-1 coverslip that is inverted and suspended over the concavity of depression slide. True motility must be differentiated from Brownian movement. Motile bacteria show directional movement and change in position relative to each other; when Brownian movement is the cause of motion, they maintain the same relative position. Motility B medium with tetrazolium also used for demonstrating motility of NFGNB. Flagellar stains can also be used to demonstrate motility.

Pigment Production

Pseudomonas produce water-soluble and diffusible pigments like fluorescein (pyoverdin), pyocyanin, pyorubin, pyomelanin that discolor the culture media. “Tech” and “Flo” media were developed to enhance the formation of water-soluble pigments pyoverdin and pyocyanin. These media have special peptones and an increased concentration of magnesium and sulfate ions to enhance pigment production. Pigment production also enhanced by growing the organism in gelatin, potato or milk-containing media and by incubating them at 25 –30^{o}C.

Hydrolysis Of Urea

Christensen’s urea agar slants used. Bacterial species like *Bordetella bronchiseptica* produce a red color change within 4hours; weak reactors may require up to 48 hours.

Nitrate Reduction

The ability of the organisms to reduce nitrate to nitrite is an important characteristic used in the identification and speciation of many microorganisms. Organisms demonstrating nitrate reduction have the capability of extracting oxygen from nitrate to form nitrite and other reduction products. The presence of nitrite in the test medium is detected by the addition of alpha-naphthylamine and sulphanilic acid which leads to the development of red color. If red color do not develop, either nitrate has not been reduced or reduction is beyond the nitrite stage to the formation of other compounds or to nitrogen gas (denitrification). The appearance of red color on addition of small quantity of zinc dust indicates the residual presence of nitrate, denoting a negative test; absence of color indicates nitrate has been reduced beyond nitrite, indicating the original test was positive.

Denitrification of Nitrates And Nitrites

Certain nonfermenters have the capability of reducing nitrate or nitrite or both to gaseous nitrogen. Nitrate-nitrite broth with an inverted Durham tube may be used. Because the media contains no carbohydrate, any gas that is formed is derived from nitrate or nitrite, indicating a positive reaction.

Indole Production

An enriched tryptophan –containing media, usually heart infusion broth may be needed. Because only small quantities of Indole are formed by some NFs, extraction of culture media by layering a small quantity of xylene or chloroform on the surface may be helpful. The appearance of fuchsia red color at the surface of medium with the reagent (kovac or Ehrlich reagent) indicates indole formation and a positive test. One organism, Delftia

acidovorans, produces a distinctive “pumpkin orange” indole reaction owing to the formation of anthranilic acid rather than indole from tryptophan.

Citrate Utilisation

A well isolated colony is picked from the surface of a primary inoculation plate and inoculated as a single streak on the slant surface of Simmon’s citrate medium and incubated at 35^{o}C for 24 to 48 hours. Development of blue color indicates a positive test.

Decarboxylation

Moeller decarboxylase medium is used. The development of alkaline purple color, following inoculation with the test organism and incubation at 35^{o}C for 24-48 hours indicates a positive test result.

Esculin Hydrolysis

This is used primarily as a differential characteristic to distinguish between the two Brevundimonas species and some of the yellow pigmented pseudomonads. An esculin medium without bile is used for nonfermenters, because some of them are inhibited by bile. Esculin agar slants are inoculated with an unknown isolate and incubated at 35^{o}C for 24-48 hours. Esculin in the medium fluoresces when observed with a wood’s lamp. When esculin is hydrolysed, the medium turns reddish –black and fluorescence is lost, indicating a positive test.

CHARACTERISTICS OF INDIVIDUAL ORGANISMS

PSEUDOMONADS

The Genus pseudomonas and closely related genera which were formerly placed in the Genus pseudomonas are referred to as pseudomonads. Pseudomonads are straight or slightly curved, aerobic, gram-negative bacilli motile by means of polar flagella and utilize glucose and other carbohydrates oxidatively and are usually cytochrome oxidase positive. Most are saprophytes found widely in soil, water and other moist environments. [31]

Molecular analysis led to revised taxonomic classification and many species have been reallocated to new genera which includes Burkholderia, Comamonas, Stenotrophomonas, Ralstonia and Brevundimonas. [24]

Palleroni separated pseudomonads into five ribosomal RNA homology groups based on r RNA-DNA homology studies.

Gilardi on the other hand separated pseudomonads into seven major groups based on phenotypic characteristics

- Fluorescent
- Stutzeri
- Alcaligenes
- Pseudomallei
- Facilis-delafieldii
- Acidovorans
- Diminuta

Pseudomonas species included in rRNA group 1 includes 3 groups

- ❖ Fluorescent group
- ❖ Stutzeri group
- ❖ Alcaligenes group

Fluorescent Group

The species within this group are characterized by the production of water-soluble pigment pyoverdin that fluoresces white to blue-green under UV light. This group includes *P.aeruginosa, P.fluorescens* and *P.putida*. Although all 3 species produce pyoverdin, only *P.aeruginosa* produces the distinctive blue water-soluble pigment pyocyanin. [30]

P.aeruginosa is the species most commonly associated with human disease. [24] There are several reasons for the prominence of *P.aeruginosa* as a human pathogen

- ❖ Its adaptability
- ❖ Its innate resistance to many antibiotics and disinfectants
- ❖ Its armoury of putative virulence factors
- ❖ An increasing supply of patient's compromised by age, underlying diseases or immunosuppressive therapy. [24]

P.aeruginosa produces a characteristic appearance on Blood agar plate(BAP) and the colonies have an alligator skin appearance and exhibits a metallic sheen with beta-hemolysis. Rapid identification in culture can be made by

- ❖ Typical colony morphology
- ❖ Production of diffusible pigments
- ❖ Presence of fruity odour
- ❖ Positive oxidase.

P.aeruginosa infection is prevalent among patients with burns, cystic fibrosis, acute leukemia, organ transplantation and intravenous drug addicts.[10] Infection commonly occurs at any site where moisture tends to accumulate-tracheostomies, indwelling catheters, burns, external ear and weeping cutaneous wounds. *P.aeruginosa* also causes urinary tract infections and lower respiratory tract infections, the later can be severe and life threatening in immunocompromised patients.[30] The organism also causes keratitis. Analysis of bacterial keratitis reveals that Pseudomonas species is the second most important cause of bacterial keratitis in India after gram-positive bacteria.[48]

P.aeruginosa produces several substances that are thought to enhance the colonization and infection of host tissues. These substances, together with the variety of virulence factors including lipopolysaccharide, exotoxin A, leucocidin, extracellular slime, proteases, phospholipases and several other enzymes make *P.aeruginosa* the most clinically significant bacteria among NFB. [30] An unususal mucoid morphotype of *P.aeruginosa* is recovered from respiratory secretions of patients with cystic fibrosis which is due to the production of large amounts of polysaccharide called alginate. The production of alginate is associated with poor prognosis and high mortality rates among patients with cystic fibrosis. [23]

P.fluorescens and *P.putida* occur in water and soil and may exist in water sources in hospital environment. Both may exist as normal pharyngeal flora and are rare opportunistic pathogens. Both the species fail to grow at 42^{o}C as *P.aeruginosa*. They produce only pyoverdin and not pyocyanin. Another character in which they differ from *P.aeruginosa* is that they do not deaminate acetamide. These two species differ from each other in gelatin hydrolysis where *P.flourescens* gives a positive reaction ; *P.putida* gives a negative reaction.[30] *P.putida* has been reported to cause catheter-related sepsis in patients with cancer and septic arthritis. [37]

Treatment of *P.aeruginosa* infection is difficult because it express innate resistance to many antibiotics. An alarming increase in resistance to various antimicrobial agents has been reported from India and abroad.[6] Increased use of broad–spectrum antibiotics, intubation of respiratory, gastrointestinal or urinary tract and intravascular catheterization are significant predisposing factors for development of antibiotic resistance.[28] They are found to be sensitive to aminoglycosides, antipseudomonal penicillin, fluoroquinolones, and third generation cephalosporins. Amikacin and ceftazidime were found to be highly effective.[6] The incidence of meropenem–resistant *P.aeruginosa* is also increasing among nosocomially infected patients in ICU.[4] The potential risk factors are previous antimicrobial drug exposure. A growing number of multidrug resistant (MDR) *P.aeruginosa* producing metallo betalactamases (MBL) is also reported. Such strains are resistant to most broad spectrum beta-lactams, aminoglycosides and fluoroquinolones and the traditional antipseudomonal antimicrobials.[2] The common form of drug resistance is mediated by lack of drug penetration (porin mutation and efflux pump) and/or cabapenem–hydrolysing beta-lactamases. Based on molecular studies, carbapenem-hydrolysing enzymes

are classified into four groups A,B,C,D. The metallo betalactamases are enzymes requiring divalent cations as cofactors for enzyme activity, being inhibited by the action of a metal ion chelator.[26] There are reports of MBL production in *P.aeruginosa* from various countries like Brazil, Korea, Singapore and France. MBL was first reported as a zinc dependent enzyme in *Bacillus cereus* in mid 1960s. A few decades later, meropenem hydrolyzing metalloenzymes were found in *Aeromonas hydrophila* and *Bacteroides fragilis*. All these enzymes were produced by chromosomal genes and at first recorded only from single clinical isolates. In 1991, Japan reported the first plasmid mediated MBL in *P.aeruginosa*. Apart from *P.aeruginosa*, other bacteria like *Serratia, Klebsiella pneumonia, Escherichia coli, Enterobacter aerogenes, Enterobacter cloacae, Citrobacter freundii, Proteus vulgaris, P.putida, Acinetobacter and Alcaligenes xylosoxidans* were also shown to produce MBL. These carbapenems may be class B MBL(VIM,IMP) or class D oxacillinases(OXA-23 to OXA-27) or class A clavulanic acid inhibitory enzymes(SME,NMC,IMI,KPC). They may be chromosomally or plasmid mediated and therefore possess a threat of spread of resistance by gene transfer among GNB. [43] Since carbapenem resistance is mediated by several mechanisms, cross-resistance is commonly seen among related antibiotics. Although there are various specific tests to detect the underlying mechanism of carbapenem resistance, Kirby-bauer disc diffusion test is a simple, easy to perform and cost-effective test which can be conveniently used to screen carbapenem resistance. These strains also remain resistant to several other antibiotics including penicillins, cephalosporins, quinolones, aminoglycosides and third generation cephalosporins including ceftazidime and cefotaxime. Thus, they may be ESBL producers as well. These MBLs effectively hydrolyse all betalactams except Aztreonam in vitro. This poses serious problems in

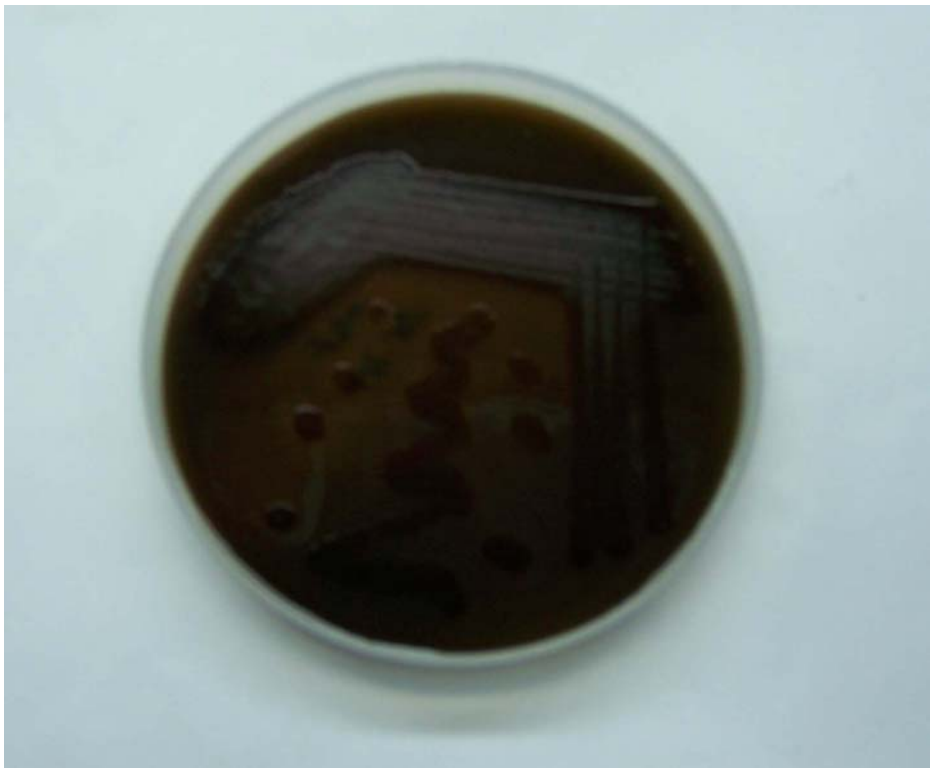
choosing the right antibiotic for treatment of sick patients admitted to ICU.[43] This disturbing situation could be attributed to the increased use of antibiotics which has to be controlled by strict antibiotic policy. Various strategies such as strict infection control measures, judicious prescribing of antibiotics, antibiotic resistance surveillance programs and antibiotic cycling must be tried. Regular monitoring and documentation of carbapenem resistance is therefore crucial in developing strategies to control infections due to these bacteria in patients admitted to ICU. Therefore, detection of MBL-producing gram negative bacilli especially *P.aeruginosa* is crucial for the optimal treatment of patients particularly in critically ill and hospitalized patients and to control the spread of resistance.

Extended spectrum metallo-betalactamases found in P.aeruginosa[17]

Enzyme(s)	Country where strains where found	Emodement Site	Associated Phenotype, by drug						Inhibition by	
			Carb-Tic	Pip-Azl	Czid	Cpm-Cpr	Atm	Imi-Mero	Clv	Taz
PER-1	Turkey mostly, Italy, France, Belgium	Plasmids (or) Chromosome	R	r	R	R	R	S	Strong	Weak
OXA-11,-14, -16, -19, -28	Turkey (OXA-11, -14&-16) France (OXA – 19 & -28)	Integrins in Plasmids (or) Chromosome	R	R	R	R	R	S	Weak	Weak
OXA – 15	Turkey	Plasmids	R	R	R	R	R	S	Weak	Weak
IMP -1 to -8	Japan (IMP-1) Canada (IMP-7)	Integrins in Plasmids (or) Chromosome	R	R	R	R	S	r/R	No	No
VIM types	Italy (VIM-1) France, Greece, Korea (VIM-2), Taiwan (VIM-8)	Integrins in Plasmids (or) Chromosome	R	R	R	R	S	r/R	No	No

Azl-Azlocillin / Carb – Carbenicillin / Clv – Clavulanate / Cpm - Cefepime / Cpr – Cefpirome / Imi – Meropenem / Czid – Ceftazidime / Mero – Meropenem / Pip – Piperacillin / Taz – Tazobactam / Tic - Ticarcillin / r- reduced susceptibility / R- frank resistance / S – Susceptible /

Colonies of P.aeruginosa on Nutrient agar



Brown Pigmented Pseudomonas

Typing Methods

Typing procedures are useful for epidemiology, that is to establish the origin of strains causative of infections and are very important to guide treatment in environments of limited dimensions.

Bacteriocin Typing

Bacteriocins are proteins produced by some strains that are lethal against the cells of other strains of the same species. Pyocins, bacteriocins produced by *P.aeruginosa*, can be used to classify *P.aeruginosa*. There are three morphological types of pyocins. The R type resembles the tails of T-even coliphages and the F types are flexuous filaments. The third type, the S type, has low molecular weight, diffuses through the agar and has no discernible details under the electron microscope. The pyocin produced by an unknown strain is tested against a series of indicator strains. This is the most common method. In a variation, the pyocin sensitivity of the unknown is measured, instead of its production. Non-fluorescent pseudomonads are not sensitive to pyocins, but fluorescent members may be so. [54]

Serologic Typing

The serological typing is based on the reaction of a heat stable O-antigen with a set of antisera initially proposed by Habs in 1957. Habs worked with 70 strains of bacteria and came up with 12 somatic groups, forming the basis of the International Antigenic Typing Scheme(IATS). Later there were several proposals to extend this number to reach 17 by addition of a few new small groups, but they were never accepted internationally. The antisera are prepared against live or boiled cell suspensions and in the agglutination reaction, boiled or autoclaved bacteria can be used. [54]

DNA Restriction Analysis

The endonucleases that are used in this type of analysis can be those that cut the DNA frequently or those that donot do so, a property that depends on the composition of the DNA and on the enzyme specificity. Pseudomonas is a taxon whose DNA is relatively rich in G+C pairs and will give more fragments after digestion with an enzyme having a high affinity for this configuration than with adenine and thymidine (A+T). For macro-restriction genome analysis, few bands are obtained by using enzymes that attack infrequently the DNA and in doing so, the fragments will be of large size and fewer in number. Their separation by electrophoresis will require special instrumentation. [54]

Vaccine Strategies against P.aeruginosa infection in the lung

P.aeruginosa is an environmentally ubiquitous, extra cellular opportunistic GNB that causes significant morbidity and mortality to a disproportionately high degree for infections with this bacteria compared with other GNB. Patients at particular risk of infection are those with compromised respiratory function, in intensive care support and taking immunocompromising pharmaceutical agents. Once acquired, infection is difficult to eradicate with chemotherapy. Initial studies in an acute animal model clearly demonstrated that mucosal immunization with a killed whole bacterial cell preparation could induce protective immune responses in the lung. Subsequent studies have shown that the protective immune mechanisms were dependent on antigen specific CD4+ T Cells, the activations of alveolar macrophages, the recruitment and activation of polymorphs, predominantly neutrophils, the controlled secretion of TNF-alpha, IL-1 & IFN gama and the presence of antibody. A pre-clinical human

trial of an oral whole killed cell preparation has been completed with no adverse side effects. Preliminary analysis of the results has demonstrated that after oral vaccination, specific lymphocyte responses were observed to *P.aeruginosa*. [15]

Characteristics of fluorescent group

Test	P.aeruginosa	P.fluorescens	P.putida
Oxidase	+	+	+
Motility	+	+	+
Pyoverdin	+	+	+
Pyocyanin	+	-	-
OF-glucose	A	A	A
Acetamide	V	+	+
Growth at 42 ^{O}C	+	-	-
Nitrate reduction	V(74)	V(19)	-
Arginine	+	+	+

+ , 90% (or) more strains positive / - , 90% (or) more strains negative / V , 11 – 89 % of strains positive / A , acid reaction / ( ) numbers in the parenthesis are % of strains giving positive reactions.

Stutzeri Group

The organism in this group – *P.stutzeri, P.mendocina* and CDCgroup Vb-3. These organisms are all soil denitrifiers and can grow anaerobically in nitrate containing media with the production of Nitrogen gas. They are motile by polar monotrichous flagella.[30] They have been recovered from humus, manure, sewage, stagnant water, baby formulas, hospital equipments, eye cosmetics and various clinical specimens.[22,40] It has been rarely associated

with infections like otitis media, conjunctivitis, pneumonia, infections of traumatic wounds and meningitis in Human Immunodeficiency Virus(HIV) patients. They are susceptibile to most antibiotics. [30]

Characteristics of stutzeri group

Test	P.stutzeri (VB-1)	P.mendocina (VB – 2)	CDC Group (VB – 3)
Oxidase	+	+	+
Motility	+	+	+
OF-glucose	A	A	A
OF – maltose	A	-	-
Nitrate reduction	+	+	+
Nitrate to gas	+	+	+
Arginine	-	+	+
Polymyxin B	S	S	S

+ , 90% (or) more strains positive / - , 90% (or) more strains negative / A , acid reaction / S - Susceptible.

Alcaligenes Group

Organisms in this group are characterized by being assacharolytic or only weakly saccharolytic in OFglucose medium. Members of this group includes *P.alcaligenes, P.pseudoalcaligenes* and Pseudomonas species CDC group 1.[30] There have been reports of *P.alcaligenes* causing eye infections, empyema and one case of fatal endocarditis. [55]

Characteristics of alkaline Pseudomonads

Test	P.alcaligenes	P.pseudo alcaligenes	Comamonas terrigena	C.testosteroni	Delftia acidovorans	Brevundimonas diminuta	B.vesicularis
Oxidase	+	+	+	+	+	+	+
Motility	+	+	+	+	+	+	+
OF-Glucose	Alk	Weak	Alk	Alk	Alk	Weak	Weak
Nitrate reduction	V (16)	+	+	+	+	-	-
Nitrate to gas	-	-	-	-	-	-	-
Indole	-	-	-	-	Orange	-	-

+ , 90% (or) more strains positive / - , 90% (or) more strains negative / V , 11 – 89 % of strains positive / Alk - alkaline reaction / ( ) numbers in the parenthesis are % of strains giving positive.

ACINETOBACTER

The Genus Acinetobacter is currently classified in the family Moraxellaceae and consists of bacteria that are non-motile, oxidase negative, gram-negative coccobacilli. Currently there are atleast 25 genomospecies in this genus. Genomospecies 1 is the type species and is named *A.calcoaceticus*. Genomospecies 2 is named as *A.baumannii* and Genomospecies8 is *A.lwofii* which are the commonly isolated species of Acinetobacter. *A.baumannii* is the second most frequently isolated non-fermenter from clinical samples especially from patients in ICU next to P.aeruginosa.[32] *A.baumannii* is a water organism and often isolated from

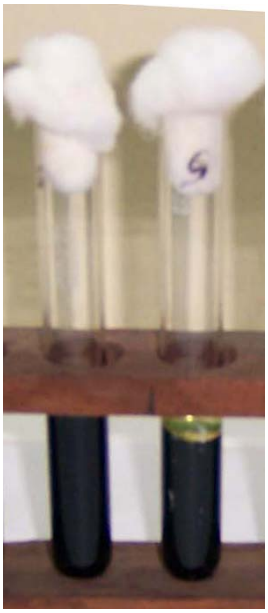
hospitalized patient's sputum or respiratory secretions, wound and urine.[1] Acinetobacter infections are uncommon but can manifest as nosocomial pneumonia, infections associated with continous ambulatory peritoneal dialysis or catheter associated bacteriuria. The characteristics by which presumptive identification can be made are

- Appear as cocci or coccobacilli in gram stain
- Grow well on MacConkey agar- colonies have a slightly pinkish tint
- Donot produce cytochrome oxidase
- Exhibit rapid utilization of glucose with the production of acid
- Exhibit rapid utilization of 10% lactose with the production of acid
- Are nonmotile
- Are penicillin resistant. [32]

Acinetobacter baumanii is a multidrug resistant organism sensitive to relatively few antibiotics. *A.baumannii* is inherently resistant to many antibiotics and multidrug resistant Acinetobacter is not a new or emerging phenomenon. The antibiotics that are usually effective are imepenem, meropenem, cefepime, piperacillin , tazobactam and polymyxinB. [1] The use of ceftazidime and cefotaxime was associated with an increased risk of nosocomial pneumonia with resistant strains of *A.baumannii*. Prevention of recurrent MDR *A.baumannii* infections was achieved after discontinuation of cefotaxime in ICUs. [29]

OF – Glucose of A.baumannii

OF – Glucose of A.lwofii



Biochemical reactions of Acinetobacter

TSI, Citrate, Indole, Urease, OF-Glucose

Characteristics of Acinetobacter

Test	Acinetobacter	
	A.baumannii	A.lwofii
Oxidase	-	-
Motility	-	-
Growth on Macconkey	+	+
OF-glucose	A	-
Nitrate reduction	-	-

+ , 90% (or) more strains positive / - , 90% (or) more strains negative / A , acid reaction.

STENOTROPHOMONAS

The Genus Stenotrophomonas was created in 1993 to accommodate *S.maltophilia*. Now in addition, 4 new species have been identified

- *S.africana*
- *S.nitritireducens*
- *S.acidaminiphila*
- *S.rhizophila*

S.maltophilia is a motile rod with polar multitrichous flagella and is oxidase negative and lysine decarboxylase positive.[33] *S.maltophilia* is ubiquitious and occasionally causes opportunistic infections and is emerging as an important hospital acquired pathogen. *S.maltophilia* is the third most frequently encountered non-fermenter in clinical laboratories. [49] The most common site for recovery is the respiratory tract, although in most patients these isolates do not appear to be clinically significant. Risk factors associated with death for patients with *S.maltophilia* isolate include

- ❖ Patient in ICU
- ❖ Age older than 40 yrs
- ❖ Mechanical ventilator support. [49]

S.maltophilia exhibit unique antibiotic susceptibility profile- it is inherenty resistant to most commonly used antipseudomonal drugs including aminoglycosides and many beta-lactam agents used against *P.aeruginosa*. Interestingly, *S.maltophilia* is inherently susceptible to trimethoprim-sulfamethoxazole, a drug that has no activity against *P.aeruginosa*.

The characteristics by which presumptive identification can be made are

- ❖ Good growth on blood agar and MacConkey agar
- ❖ Do not produce cytochrome oxidase
- ❖ Produce acid in OF maltose but may be negative in OF glucose
- ❖ Lysine decarboxylase positive
- ❖ DNAse positive
- ❖ Some strains show yellow pigment

Characteristics of Stenotrophomonas maltophilia and Burkholderia cepacia complex

Test	S.maltophilia	B.cepacia complex
Oxidase	-	+ (93)
Motility	+	+
Growth on Macconkey	+	+
OF-glucose	A/Weak	A
Nitrate reduction	V(42)	V (37)
Nitrate to gas	-	-
Lysine	+	+
Polymyxin B	S	R

+ , 90% (or) more strains positive / - , 90% (or) more strains negative / V , 11 – 89 % of strains positive / A – acid reaction / ( ) numbers in the parenthesis are % of strains giving positive/ S- Susceptible / R-Resistant

ALCALIGENES

Alcaligenes faecalis is the most frequently isolated member of this Genus. Members of this species produce strong alkaline reactions in all carbohydrate media. A key biochemical feature of this species is its ability to reduce nitrite but not nitrate. *A.faecalis* exist in soil and water and causes opportunistic infections like acute otitis media, urinary tract infection and bacteremia. It is often found in mixed cultures, particularly in samples of diabetic ulcers of foot and its clinical significance is difficult to determine.[33]

BURKHOLDERIA

In the past they have been referred to as Pseudomallei group. All the species in this group are easily separated from other group of Pseudomonads by the property of exhibiting resistance to polymyxin group of antibiotics (PolymyxinB and colistin). Two of the pathogenic species are *B.mallei* and *B.pseudomallei*.[33]

B.mallei is a small gram-negative coccobacilli and it is the only non-motile species in the genus. It is an obligate parasite of animals causing respiratory tract infection known as glanders. *B.pseudomallei* causes melioidosis which has been known as “Vietnamese time bomb” due to its ability to produce latent infections which can be reactivated many years after primary exposure.[16] Infections are acquired either by inhalation or direct contact through breaks in the skin.[33] Three forms of melioidosis are known

- Acute disease – presenting as septicemia with metastatic lesions
- Sub-acute disease- presenting as TB like pneumonia with cellulitis and lymphangitis
- Chronic disease- presenting as localized chronic cellulitis.

Diabetes mellitus is an important risk factor for development of bacteremic melioidosis.[53] The mortality rate is 95% in patients with acute disease who are not treated. *B.pseudomallei* is inherently resistant to many antibiotics including penicillins, first and second generation cephalosporins, macrolides, rifamycins, colistin and aminoglycosides[51]. This unusual antibiotic profile–gentamycin and colistin resistant and amoxycillin-clavulanate susceptible, in an oxidase positive gram-negative bacilli is useful for confirming the identification of *B.pseudomallei*. The organism grows readily on most of routine laboratory media and can be recovered from blood using standard blood culture technique. Selective agar either Ashdown's selective agar or *B.pseudomallei* selective agar or selective broths are recommended for isolation of *B.pseudomallei* from nonsterile body sites like sputum in a clinically suspected case of melioidosis. Commercial systems like Vitek 1&2, API 20NE, API 20E are available for identification of *B.pseudomallei*. [33]

B.cepacia

B.cepacia is a phytopathogen that causes onion bulb rot in plants and foot rot in humans. Since, early 1980s, *B.cepacia* has emerged as a cause of opportunistic human infections, particularly in patient's with chronic granulomatous disease and cystic fibrosis[41,42]. Recent taxonomic advances demonstrate that *B.cepacia* is actually a cluster of atleast 9 closely related genomic species, now called *B.cepacia* complex which includes *B.cepacia* (genomovar1), *B.multivorans* (genomovar2), *B.cenocepacia* (genomovar3), *B.stabilis* (genomovar4), *B.vietnamiensis* (genomovar5), *B.dolosa* (genomovar 6), *B.ambifaria* (genomovar7), *B.anthina* (genomovar 8) and *B.pyrrocinia* (genomovar 9). These bacteria are associated with "cepacia syndrome" manifested as severe progressive respiratory failure and

bacteremia. Disinfectants in which *B.cepacia* will grow include povidone-iodine, quaternary ammonium compounds and chlorhexidine. It also grow in distilled water with nitrogen source owing to the ability of the organism to fix CO2 from air. Selective media with bacteriostatic dyes, antibiotics or low pH have been described for selective isolation of *B.cepacia*. These include *Pseudomonas cepacia* medium(PCM) containing crystal violet, polymyxin B and ticarcillin,OFPBL medium containing polymyxinB, bacitracin and lactose and *B.cepacia* selective agar(BCSA) containing lactose, sucrose, polymyxinB, gentamicin and vancomycin. Unlike other common pseudomonads, *B.cepacia* is resistant to aminoglycoside antibiotics but is usually susceptible to trimethoprim-sulfamethoxazole, which has become the drug of choice in treating *B.cepacia* infections. [33]

B.gladioli

Formerly known as *Pseudomonas marginata*. It is primarily a plant pathogen causing flower rot in gladiolus and other plants. It is oxidase negative and produces non-fluorescent yellow colonies. It has been reported to cause human pulmonary disease and sometimes bacteremia and soft tissue infection in patients with cystic fibrosis, chronic granulomatous disease, diabetes mellitus and other immunological deficiencies.[12,50] Molecular methods are more reliable when confirmation of an isolate as *B.gladioli* is deemed necessary. The antibiotic susceptibility pattern may also serve as a clue that an organism might be *B.gladioli*, since it tends to be susceptibile to aminoglycosides, meropenem, ciprofloxacin and trimethoprim-sulfamethoxazole and resistamt to aztreonam and cephalosporins. [33]

Characteristics of Burkholderia mallei and

Burkholderia pseudomallei

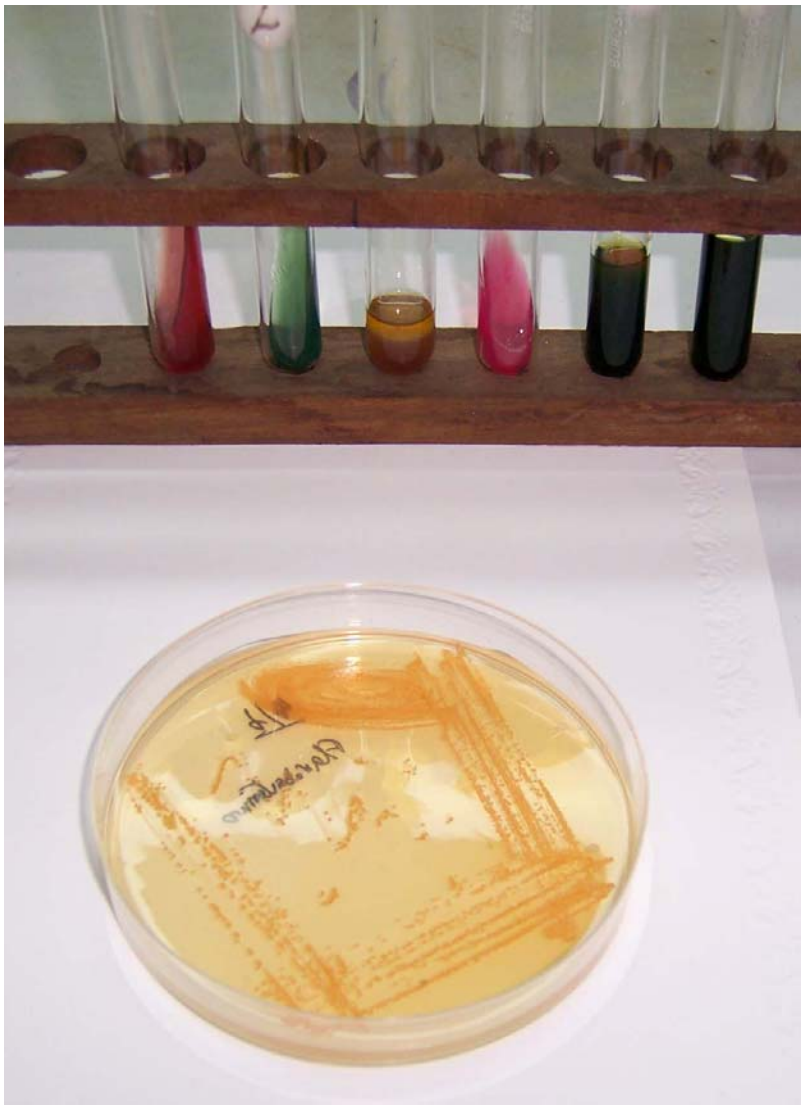
Test	B.mallei	B.pseudomallei
Oxidase	V	+
Motility	-	+
Growth on Macconkey agar	V	+
OF-glucose	A	A
Nitrate reduction	+	+
Nitrate to gas	-	+
Agrinine	+	+
Polymyxin B	R	R

+ , 90% (or) more strains positive / - , 90% (or) more strains negative / V , 11 – 89 % of strains positive / A - acid reaction / R – Resistant.

FAMILY FLAVOBACTERIACEAE

Most species in this family produce yellow-pigmented colonies on blood agar plate and all are oxidase positive. All species are non-motile and negative for nitrate reduction and most species fails to grow on MacConkey medium. The organisms previously included in the Genus Flavobacterium are now classified in the Genus Chryseobacterium, Sphingobacterium and Empedobacter.[56] *Chryseobacterium meningosepticum* is the species most often associated with significant disease in humans. It has been reported to cause pneumonia, endocarditis, wound infection, postoperative bacteremia and meningitis.[44] It is highly pathogenic for premature infants and has been associated with neonatal meningitis. They are isolated from soil, plants, water, food-stuffs, hospital water sources including incubators, sinks, tap water, haemodialysis system and saline solution.[32]

C.meningosepticum
Biochemical reactions and colonies on nutrient agar



Characteristics of indole positive group

Test	Chryseobacterium			Empedobacter
	C.meningosepticum	C.gleum	C.indologenes	E.brevis
Oxidase	+	+	+	+
Motility	-	-	-	-
Growth on Macconkey	V (26)	V (50)	-	-
OF – glucose	A	A	A	V (80)
Indole	+	+	+	+
Nitrate to nitrite	-	V (67)	V (14)	-
Nitrate to gas	NA	+	-	-
Polymyxin B	R	R	S (3%)	R

+ , 90% (or) more strains positive / - , 90% (or) more strains negative / V , 11 – 89 % of strains positive / A – acid reaction / ( ) numbers in the parenthesis are % of strains giving positive/ S- Susceptible / R-Resistant

METHODS FOR IDENTIFICATION USING CONVENTIONAL TESTS

WEYANT(CDC), GILARDI AND PICKETT IDENTIFICATION SCHEMES

If an unknown NFGNB is not *P.aeruginosa, A.baumannii* or *S.maltophilia*, additional characteristics must be determined to make a species identification. Several schemes are currently being used in clinical laboratories. The schemes designed by Pickett, Gilardi and Weyant and associates have the largest database.

CDC SCHEME-WEYANT AND ASSOCIATES

In answer to the problem of how to identify NFs in the clinical laboratory without doing all the tests that are done in a reference laboratory, Weyant and colleagues have published a three-part guide that includes

-An identification key for gram-negative aerobes

- A set of 12 identification tables
- A numerical code book by which derived biotype members can be linked to species name.

To correctly interpret the results from a given identification table, one must use the same procedures on which the reactions are based.[32]

THE GILARDI SCHEME

The Gilardi approach is based on two fundamental principles that have made it practical for use in clinical laboratories

The media and tests are readily available in most clinical laboratories and frequently the same as those used for identification of other groups of bacteria including Enterobacteriaceae,

Identification of most clinical isolates can be made in 2 stages- 1.through the use of a primary battery of media and reactions that are sufficient in the majority of cases and 2.a secondary battery, available when the first is inadequate. [32]

THE PICKETT SCHEME

Pickett was among the first to bring some order to the identification of NFs. His system was designed to identify rapidly the two most frequently recovered NFs-*P.aeruginosa* and *A.baumannii*. Pickett advocated the use of a heavy inoculum prepared in an aqueous suspension from overnight growth of bacteria that was inoculated into buffered single substrates for testing the acidification of carbohydrates the alkanization of amides and organic salts. [32]

COMPUTER AIDED SYSTEMS

BioBASE is a DOS-based computer program for computer-aided identification of microorganisms. The system enables the user to create and access hundreds of microbial databases and switch between them, depending on the primary characteristics of the microbial isolate being studied. Included with this software is the database that includes 66 taxa of NFs identified using 83 phenotypic tests.

PIBWIN(Probabilistic Identification of Bacteria for Windows)

This program provides probabilistic identification of unknown bacterial isolates against identification matrices of known strains. The program has 3 major functions

1.Identification of an unknown isolate

2.the selection identification is not achieved

3.Storage and retrieval of results. [32]

COMMERCIAL KIT SYSTEMS

Packaged kit systems have been designed for or adapted to the identification of NFGNB. These kits share many of attributes of packaged systems ingeneral. Packaged systems have found wide acceptance in clinical laboratories for the following reasons

1. their accuracy has proved to be comparable with that of conventional identification systems

2. several of the systems have a long shelf-life-6 months to 1 year, so that outdating of media, a problem particularly with conventional systems is minimized.

3. the system requires only a minimum of space for storage and incubation.

4. some of these systems are as easy or easier to use than the conventional methods. Inoculation is simple, reactions are generally clear cut within 24 hours and the availability of computer-assisted file registers make final identification easy and accurate.

These kits include various substrates like nitrate reduction, tryptophanase, glucose fermentation, arginine dihydrolase, urease, esculin hydrolysis, gelatinase and beta-galactosidase.

Inherent problems in the use of many of the currently available packaged kits for identifying NFs include the

1. tendency for organisms that exhibit weak or delayed biochemical activity to produce false negative results

2. less than optimal design of many systems for the cultivation of certain NFs

3. inclusion of some differential tests that may not be applicable to identification of NFs. [32]

Various kit systems available are

- Oxi-Ferm tube

- API 20E

- API 20NE

- Remel uni-N/F system
- Crystal Enteric/Non-fermenter system
- RapID NF plus
- Biolog system

AUTOMATED IDENTIFICATION SYSTEMS [32]

VITEK 2 SYSTEM

It is an integrated modular system that consists of a filling-sealer unit, a reader incubator, a computer control module, a data terminal and a multicopy printer. The new card contains 47 tests and the database for the new card has been expanded to 159 taxa compared with only 101 for the original vitek 2 card. Vitek 2 card correctly identified 92.4% NFs to the species level.

THE VITEK LEGACY SYSTEM

The vitek legacy system has been used with success in the identification of NFs most frequently encountered in the clinical laboratory. Correct identification to the species level at initial testing was 71.8%, improving to 92.3% after additional testing was performed as recommended by manufacturer's protocol.

THE MICROSCAN WALKAWAY-96,WALKAWAY-40 AND AUTOSCAN-4SYSTEM

It is a fully automated instrument. Walkaway-96 rapid gram-negative panel is reported to correctly identify 92.3% of non-enteric bacilli. The walkaway system correctly identified 71.4% of isolates to species level at initial testing, improving to 96% after additional tests.

THE SENSITITRE AP80 SYSTEM

It is an automated system that uses fluorescence technology to detect bacterial growth and enzyme activity. It consists of 32 biochemical tests, each test medium along with an appropriate fluorescence indicator is dried into the individual well. Results are transmitted to a computer for analysis and identification. It permits identification of GNB in as little as 5 hours, with the option of additional overnight incubation if needed or desired. Correct identification was obtained for 95.1% of all NFs tested.

THE PHOENIX SYSTEM

It is a newly developed, fully automated, identification and antimicrobial susceptibility testing system. It uses 45 biochemical substrates including 16 fluorogenic, 14 fermentation, 8 carbon source, 5 chromogenic and 2 miscellaneous substrates(urea and ornithine) with vast majority of results provided in 4 hours or less. 89.3% of correct identification is obtained with this system.

MOLECULAR METHODS

Various molecular methods like in-situ hybridization and nucleic acid amplification techniques like PCR is used for direct detection of pathogenic bacteria (like *P.aeruginosa, B.cepacia, S.maltophilia*) in clinical specimens. It demonstrated 90% sensitivity and 100% specificity compared with culture, even in the complicated milieu of respiratory specimens from patients with cystic fibrosis. Nucleic acid amplification techniques are also useful for detection of genes (those producing MBL) responsible for drug resistance.

Genotyping of *P.aeruginosa* can be done by Random Amplified Polymorphic DNA (RAPD) analysis, Pulsed-field gel electrophoresis (PFGE) and amplified fragment length polymorphism (AFLP). [52]

Genes for detection of MBL in P.aeruginosa

Multiplex PCR for detection of bla_{IMP} and bla_{VIM} MBL genes can be carried out using the IMP-DIA (forward, 5' – GGA ATA GAG TGG CTT AAT TCT C; reverse, 5' GTG ATG CGT CYC CAA YTT CAC T) and VIM – DIA (Forward, 5' – CAG ATT GCC GAT GGT GTT TGG; reverse, 5' – AGG TGG GCC ATT CAG CCA GA) Primers. [34]

Aims and Objectives

AIM AND OBJECTIVE OF THE STUDY

- To isolate non-fermentative Gram negative bacilli in various clinical samples processed from patients admitted in Intensive Care Unit in Government Stanley Hospital.
- To establish the significance of their isolation.
- To study the susceptibility pattern of these organisms to institute early and effective treatment.
- To find out the emerging pattern of resistance in these organisms by phenotypic methods.

Materials and Methods

MATERIALS AND METHODS

STUDY DESIGN : Descriptive study

The study was conducted in the department of microbiology, Government Stanley Medical College and Hospital.

STUDY PERIOD

Over a period of one year from June 2006-May 2007

SAMPLE SPECIFICATION

Samples were collected from 100 cases admitted to ICU with the following criteria

INCLUSION CRITERIA

1. All patients admitted to ICU developing infective symptoms. The patients were closely monitored for any evidence of clinical manifestations suggestive of infections viz, fever, leukocytosis, leucopenia, purulent endotracheal secretions, copious wound discharge, recent radiological infiltrates in chest X-ray etc.

2. Non-fermenters isolated from two consecutive samples were included in the study.

EXCLUSION CRITERIA

1. Patients who were admitted to the ICU following prior stay in other wards of the hospital.

2. Patients admitted in the ICU following more than 24hrs of stay in neighouring hospitals.

3. Non-fermenters not isolated from two consecutive samples were excluded from the study.

METHODOLOGY

SPECIMEN

Appropriate clinical sample based on the clinical manifestations like pus, sputum, blood, urine, CSF, endotracheal aspirates were collected taking adequate aseptic precautions. A total of 135 samples were collected from 100 patients which includes - pus(33), sputum(25), urine(31), blood(25), endotrcheal aspirate(10), CSF(5), pleural fluid(5), bronchoalveolar lavage(1).

SPECIMEN COLLECTION

Sputum

The patients were instructed what constituted a proper sputum specimen and explained how it should be produced from the depth of chest. The patient was asked to expectorate 5-10ml of sputum directly into a sterile container, as far as possible without spoiling the outer surface. If spoiled, after tightly closing the container the outer surface was wiped with cotton dipped in 70% alcohol.

Urine- clean catch, midstream urine in non-catheterised patients

In case of indwelling catheter, tubing was clamped off above the port to allow collection of freely voided urine. The catheter port (or) wall of the tubing was then cleaned vigorously with 70% ethonal and urine was aspirated via a

needle and syringe; the integrity of the closed drainage system was maintained to prevent the introduction of microorganisms.

Pus Specimen

The wound margins were decontaminated with spirit and a deep wound swab taken, taking care not to touch the adjacent skin margins. The swab was then introduced into sterile test tube and transported immediately to the diagnostic laboratory. Two swabs were collected from each wound and one was subjected to gram stain and the other for culture studies.

Blood Sample

The vein from which blood is to be withdrawn was selected and the skin site was disinfected with betadine and 70% alcohol to reduce the risk of introducing contaminants. About 5 ml of blood was collected and transferred to 50 ml of Brain-Heart infusion broth.

Cerebrospinal Fluid

CSF was collected by aseptically inserting the needle into the sub-arachnoid space, usually at the level of lumbar spine. A minimum of 5-10 ml was collected into a sterile test tube. It was delivered immediately to the laboratory for detection of microorganisms by centrifugation and culture.

Bronchoalveolar Lavage

A deeper sampling of desquamated host cells and secretions can be obtained by BAL. During this procedure, a high volume of saline (100-300ml) is infused into a lung segment through the bronchoscope to obtain cells and protein of pulmonary interstitium and alveolar spaces. It is estimated that

more than 1 million alveoli are sampled during this process. BAL has been shown to be safe and practical method for diagnosing opportunistic pulmonary infections in immunosuppressed patients.

Endotracheal Aspirate

Collected in a sterile container under aseptic precautions.

PRELIMINARY ENAMINATION OF SPECIMEN

All the specimens collected were examined for macroscopic characteristics like color, consistency, odor, presence of blood and they were recorded.

MICROSCOPIC EXAMINATION

All the specimens were subjected to Gram staining and the presence of organisms, pus cells and other cells were noted.

PRIMARY CULTURE

Urine, Sputum, Pus, Endotracheal Aspirate ,Bal

Each sample was inoculated into Blood agar (BAP), Macconkey agar and Nutrient agar and incubated aerobically at 37^{o}C for 24 hours.

CSF

Two or three loopful of centrifuged sediment was placed on each culture medium and incubated aerobically at 37^{o}C for 24hours.

Blood

After 48 hours of enrichment in brain-heart infusion broth, subculture was made on BAP and MacConkey agar.

BASIC APPROACH FOR IDENTIFICATION OF NON-FERMENTERS

1. All non-lactose fermenting colonies on MacConkey with smooth colonies on BAP were considered significant.

2. Tiny colonies on BAP with poor or no growth on MacConkey were considered significant.

These isolates were then subjected to

-Gram stain and

- Inoculated in Triple sugar iron agar (TSI)

All GNB which lacks acid production in TSI manifested as alkaline slant and an alkaline deep reaction AK/AK or alkaline slant and no change reaction AK/- considered as NFGNB and preserved for further identification tests.

IDENTIFICATION OF ISOLATES

Once a ten isolates of NFGNB were isolated, they were subjected to various biochemical tests for speciation. The tests include

- ❖ Motility
- ❖ Oxidase
- ❖ Indole production
- ❖ Hugh-Leifson Oxidation-Fermentation test

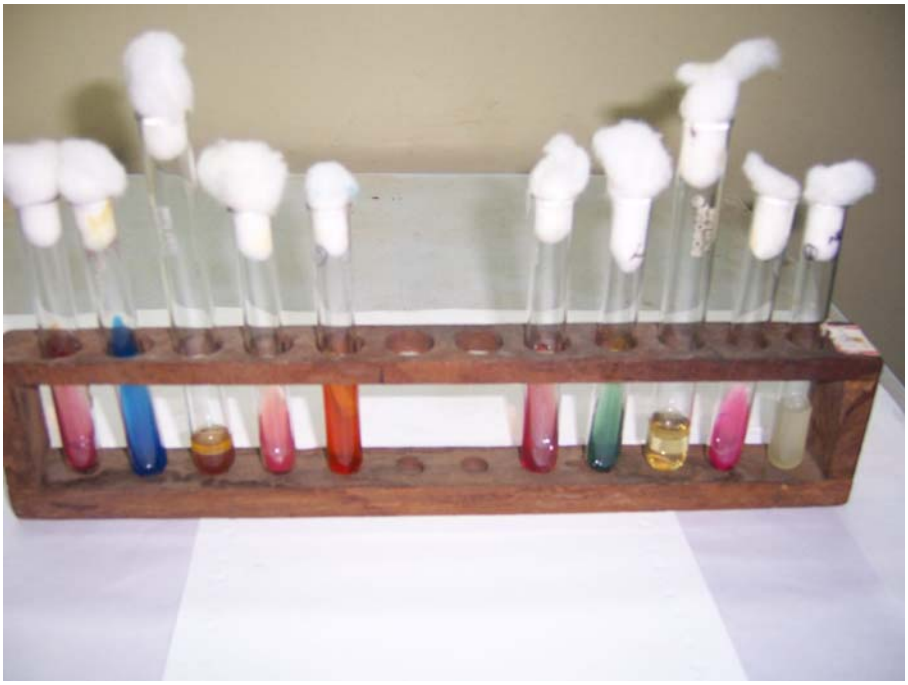
Biochemical reactions for identification of non-fermenters

TSI / Citrate / Indole / OF-Glucose / OF – Lactose / OF – Sucrose /

- OF- Maltose / OF- Mannitol / OF – Xylose / Nitrate reduction / Lysine, Arginine, Ornithine Decarboxylase

Biochemical reactions

Pseudomonas **Acinetobacter**



Pseudomonas – TSI- K/K, Citrate – Positive, Indole – Negative , Urease – Negative, Nitrate reduction – Positive

- Acinetobacter – TSI – K/K, Citrate – Negative, Indole – Negative, Urease – Positive, Nitrate reduction - Negative

OF-Reactions

Pseudomonas

Acinetobacter

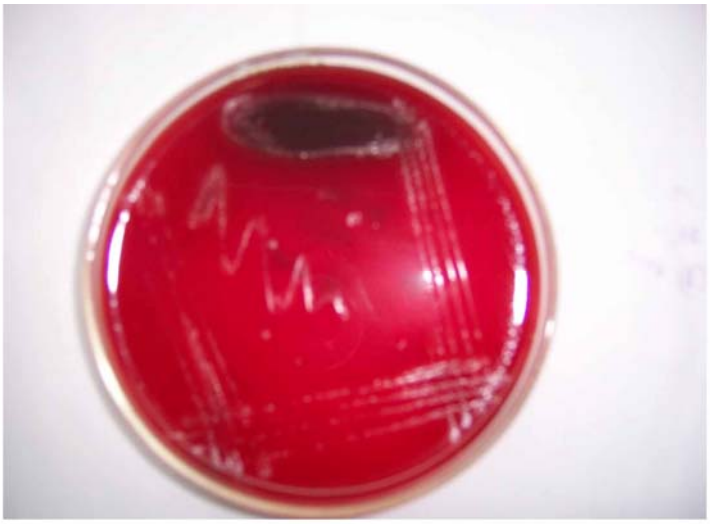
OF-Glucose, OF-Lactose, OF-Sucrose, OF-Maltose,
OF-Mannitol, OF-Xylose

Shewanella Putrefaciens

Biochemical reactions

OF-Glucose, OF-Lactose, OF-Sucrose, OF-Maltose, OF-Mannitol, TSI, Citrate, Indole

Colonies on Blood agar



- ❖ Lysine, Arginine, Ornithine decarboxylase test (LAO)
- ❖ Citrate utilization
- ❖ Nitrate reduction
- ❖ Urease production
- ❖ Pigment production

ANTIMICROBIAL SUSCEPTIBILITY TESTING

After isolating and identifying the organism their antimicrobial susceptibility testing was performed by Kirby-Bauer disc diffusion method. The test was performed on Muller-Hinton agar (MHA) using commercially available discs. Turbidity of actively growing broth culture was adjusted so that it is optically comparable to that of 0.5 McFarland standard. A sterile cotton swab was dipped into the suspension and was pressed firmly on the side wall of test tube above the fluid level to drain the excess fluid. The dried surface of MHA was inoculated by streaking the swab over the entire sterile agar surface. This procedure was repeated by streaking two more times rotating the plate approximately 60 degrees each time to ensure an even distribution of inoculum. The predetermined battery of antimicrobial discs which included Ciprofloxacin, Amikacin, Gentamycin, Piperacillin-Tazobactam, Meropenem and Ceftazidime was dispensed onto the surface of inoculated agar plate. The discs were evenly distributed so that they were not closer than 24mm from center to center. The plates were incubated aerobically at 37^{o}C for 16 to 18 hours. After this period, each plate was examined and if the plate was satisfactorily streaked and the inoculum was correct, the resulting zones of inhibition will be uniformly circular and there will be confluent lawn of growth. The diameter of zones of inhibition was measured including the diameter of disc. The zone size were interpreted by

referring to NCCLS standards and reported as either susceptible, intermediate or resistant.

Controls used with each batch- *P.aeruginosa* ATCC 27853.

DETERMINING THE SIGNIFICANCE OF THE ISOLATES

The pathogenicity and significance of all the NFs isolated from the patients was confirmed by repeat isolation of the organism by obtaining a second sample from the same patient.

DETECTION OF METALLO BETALACTAMASES

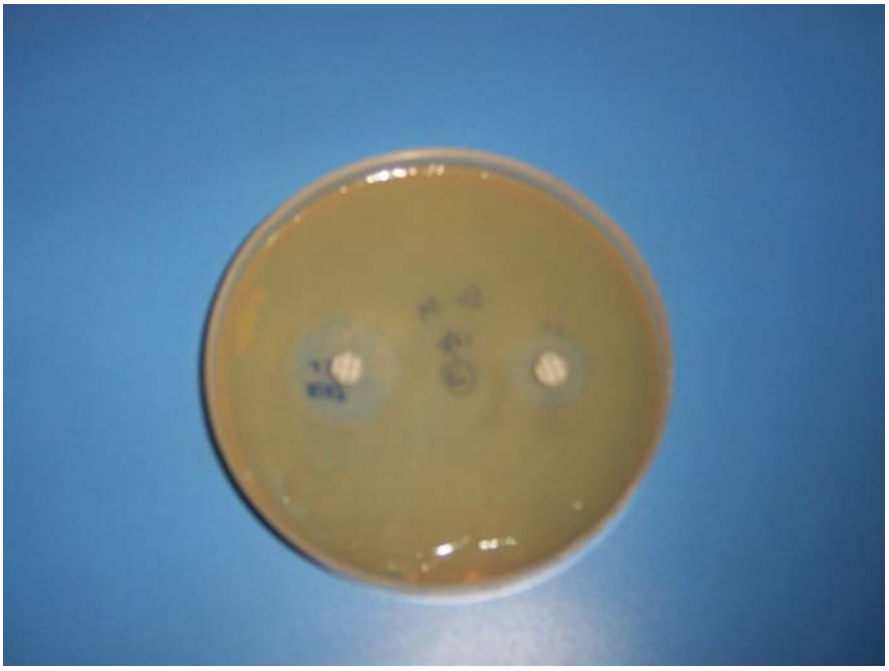
Double disc synergy using meropenem and EDTA discs or ceftazidime and EDTA discs was used to screen MBL.

Zone enhancement with EDTA impregnated meropenem and ceftazidime discs

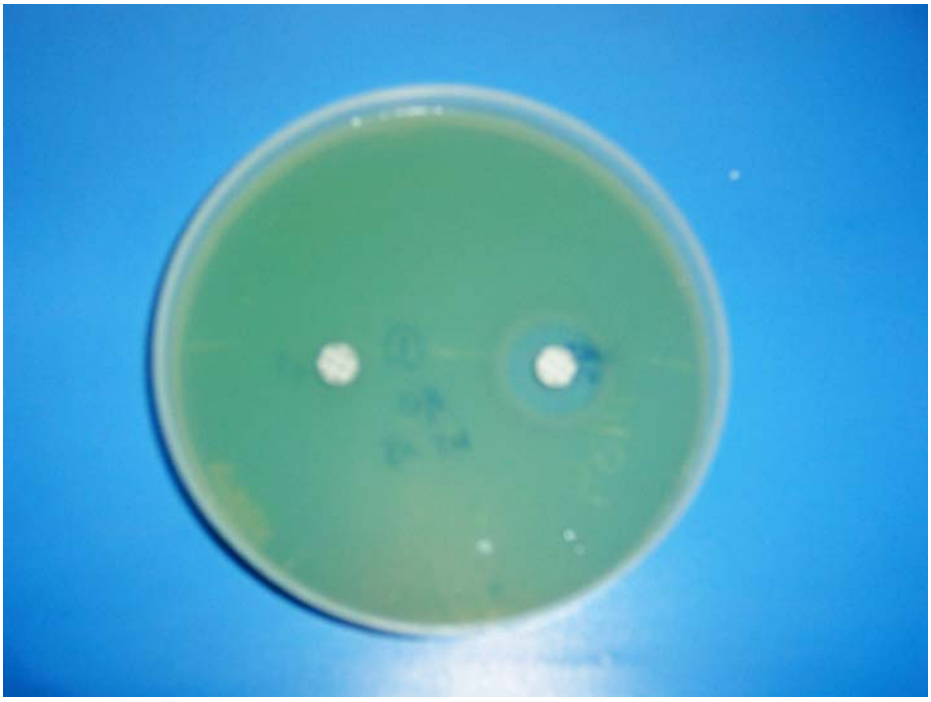
Test organisms were inoculated on to plates with MHA as recommended by NCCLS. A 0.5M EDTA solution was prepared by dissolving 186.1 g of EDTA.2 H_20 powder in 1000 ml distilled water and adjusting the pH to 8.0 by using NaOH. The mixture was sterilized by autoclaving. Two 10 microgram of meropenem disc and two 30 microgram of ceftazidime disc were placed on the surface of an agar plate and EDTA solution was added to one of them to obtain a desired concentration of 750 micrograms. The inhibition zones of meropenem, ceftazidime and meropenem EDTA and ceftazidime EDTA discs were compared after 16-18 hrs of aerobic incubation in air at 35^{o}c.

To test the stability of the EDTA added discs, an EDTA solution was added to 10 microgram meropenem disc and 30 microgram ceftazidime discs to obtain a concentration of 750 micrograms. The discs were dried immediately in an incubator and stored at $4^{o}C$ (or) at $– 20^{o}C$ in an airtight vial without any desiccant. The inhibition zones produced for MBL-positive and MBL-Negative isolates were compared after storage of discs. [35,59]

Zone enhancement with Ceftazidime-EDTA disc



Zone enhancement with Meropenem -EDTA disc



Results

RESULTS

The study was performed during June 2006 to May 2007 at the Department of Microbiology, Government Stanley Medical College and Hospital. The study included 135 samples from 100 patients admitted to Intensive Care Unit.

TABLE 1

Percentage of NFGNB among various Isolates

Total No. of Samples	Total No. (%) of NFGNB	Total No. (%) of FGNB	Total No. (%) of other isolates
135	22 (16.3 %)	65 (48.15 %)	33 (24.44 %)

GNB – Gram Negative Bacilli / NFGNB – Nonfermenting GNB / FGNB – Fermenting GNB/ Other isolates -Gram Positive Cocci (GPC), Diphtheroids, candida.

Out of total 135 samples, NFGNB accounted for 16.3 % of isolates. This falls within 95% CI (10.8 – 23.3). FGNB accounted for 48.15% and 24.44 % of isolates were found to be GPC, Diphtheroids and candida. The remaining samples were culture negative (11.11%).

Percentage Of Various Isolates

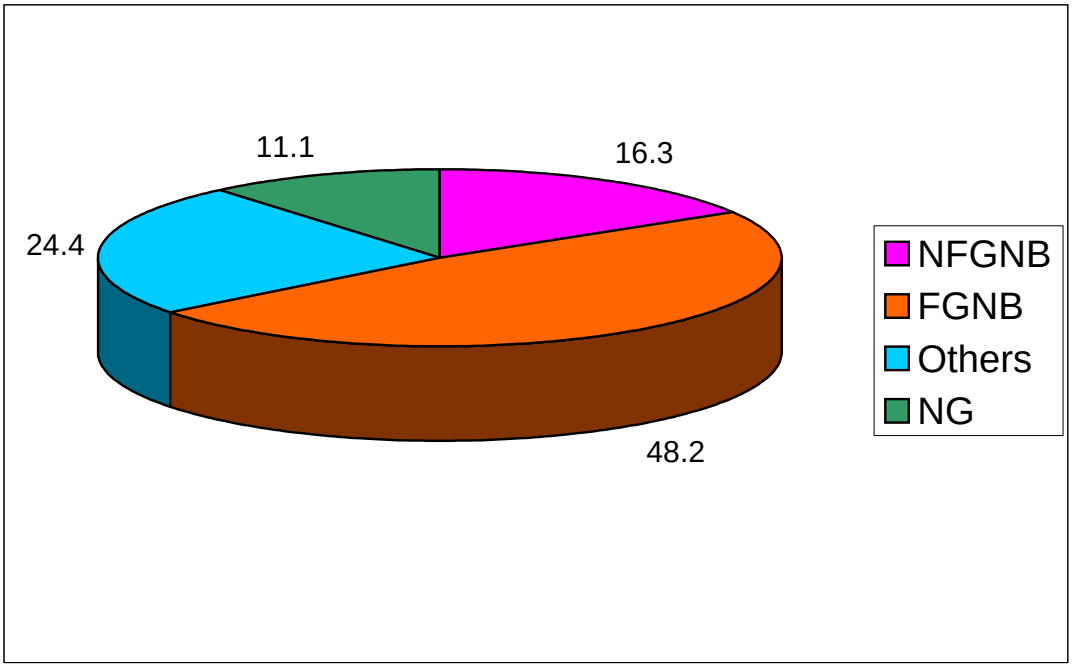


TABLE - 2

Total number of isolates from various Clinical Samples (n = 135)

Sl	Sample	Total No.of Samples	Total No. of GNB Isolates	Total No. of NFGNB	Total No. of FGNB	Total No. of other Isolates	NG
1.	Urine	31	24	5	19	3	4
2.	Sputum	25	12	3	9	12	1
3.	Pus	33	24	6	18	9	-
4.	Blood	25	15	3	12	6	4
5.	Cerebro spinal fluid (CSF)	5	1	-	1	-	4
6.	Broncho alveolar lavage (BAL)	1	1	1	-	-	-
7.	Endotracheal aspirate	10	7	4	3	3	-
8.	Pleural fluid	5	3	-	3	-	2

GNB – Gram Negative Bacilli / NFGNB – Nonfermenting GNB / FGNB – Fermenting GNB / Others – (GPC), Diphtheroids, candida, NG-No Growth

Out of 135 samples, 22 NFs were obtained which accounted for 16.3% of isolates.

TABLE – 3
Total No.of various NFGNB Isolated from Clinical samples

Sample	Total No.of NFGNB	P.aeruginosa	P.fluorescens	A.baumannii	A.lwofii	C.meningosepticum	S.putrefaciens
Pus	6	3	1	-	-	1	1
Sputum	3	3	-	-	-	-	-
Urine	5	4	1	-	-	-	-
Blood	3	2	-	1	-	-	-
Endotracheal Aspirate	4	3	1	-	-	-	-
BAL	1	-	-	-	1	-	-
Total	22	15	3	1	1	1	1

P.aeruginosa was the commonest isolates irrespective of the source and constitutes 68.18%. The next common isolate was *P.fluorescens* which constitutes 13.63%. The other NFs isolated were *A.baumannii*, A.lwofii, *Chryseobacterium meningosepticum* and Shewanella putrefaciens which constitutes 4.54% respectively.

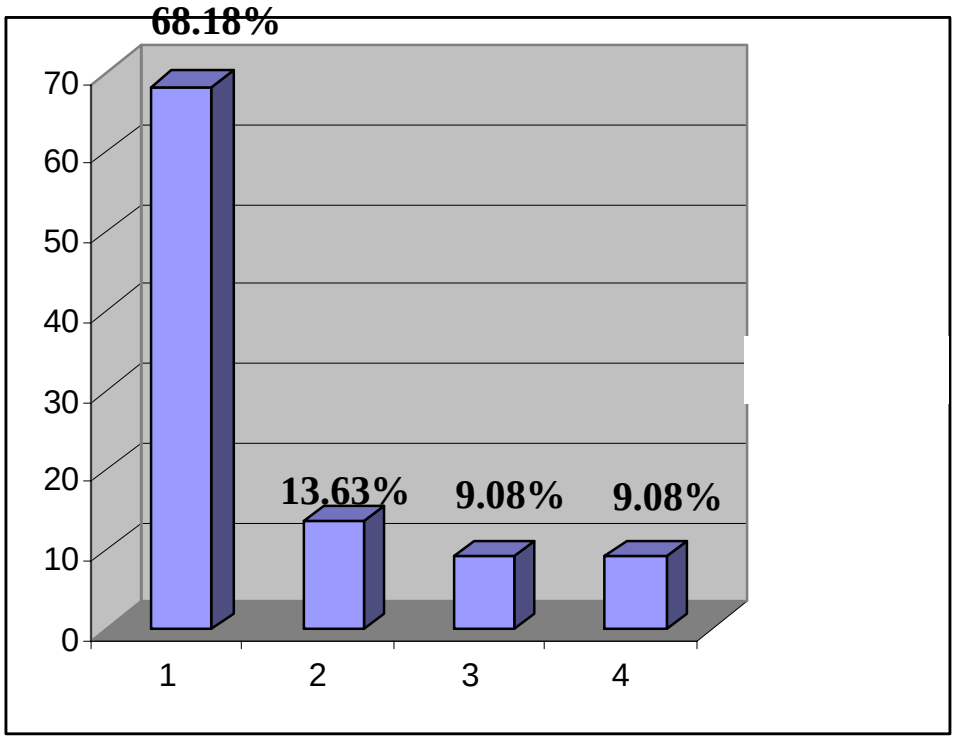
TABLE – 4

Percentage of various NFGNB among the Isolates (n = 22)

Organism	Total No.Isolate	Percentage
P.aeruginosa	15	68.18
P.fluorescens	3	13.63
A.baumannii	1	4.54
A.lwofii	1	4.54
C.meningosepticum	1	4.54
S.putrefaciens	1	4.54

1 out of 9 samples collected from ICU were infected with *P.aeruginosa*. Infection with *P.fluorescens* was found in 1 out of 45 samples while only 1 out 135 samples were infected with *Acinetobacter* and other NFs.

DISTRIBUTION OF VARIOUS NFGNB AMONG THE ISOLATES



1. P.aeruginosa
2. P.fluorescens
3. Acinetobacter species
4. Others (Chryseobacterium and Shewanella)

TABLE – 5

Antibiotic sensitivity profiles of various NFGNB (n = 22)

Organism	Gentamycin	Amikacin	Ciprofloxacin	Piperacillin-Tazobactam (P/T)	Cefotaxime	Ceftazidime	Meropenem
Pseudomonas (n = 18)	11	15	12	10	8	12	11
Acinetobacter (n = 2)	2	2	2	2	1	2	2
Others (n = 2) (Chryseobacterium & Shewanella)	1	1	2	1	1	2	2

TABLE - 6

Antibiotic Profile of Pseudomonas Isolates (n = 18)

Antibiotics	Sensitive	Intermediate	Resistant
Gentamycin	8 (44.44 %)	3 (16.66%)	7 (38.88 %)
Amikacin	13 (72.22%)	2 (11.11%)	3 (16.66%)
Ciprofloxacin	12 (66.66%)	-	6 (33.33%)
P/T	8 (44.44%)	2 (11.11%)	8 (44.44%)
Cefotaxime	5 (27.77%)	3 (16.66%)	10 (55.55%)
Ceftazidime	9 (50%)	3 (16.66%)	6 (33.33%)
Meropenem	11 (61.11%)	-	7 (38.88%)

Table 5 & 6

Amikacin was the highly effective drug with sensitivy of 83.33%. Next effective drugs were Ceftazidime and Ciprofloxacin with sensitivy of 66.7% and 66.6% respectively. Meropenem has a sensitivy of 61.1%. Cefotaxime was the least effective drug with the sensitivy of 54.4%.

SENSITIVITY PATTERN OF PSEUDOMONAS ISOLATES

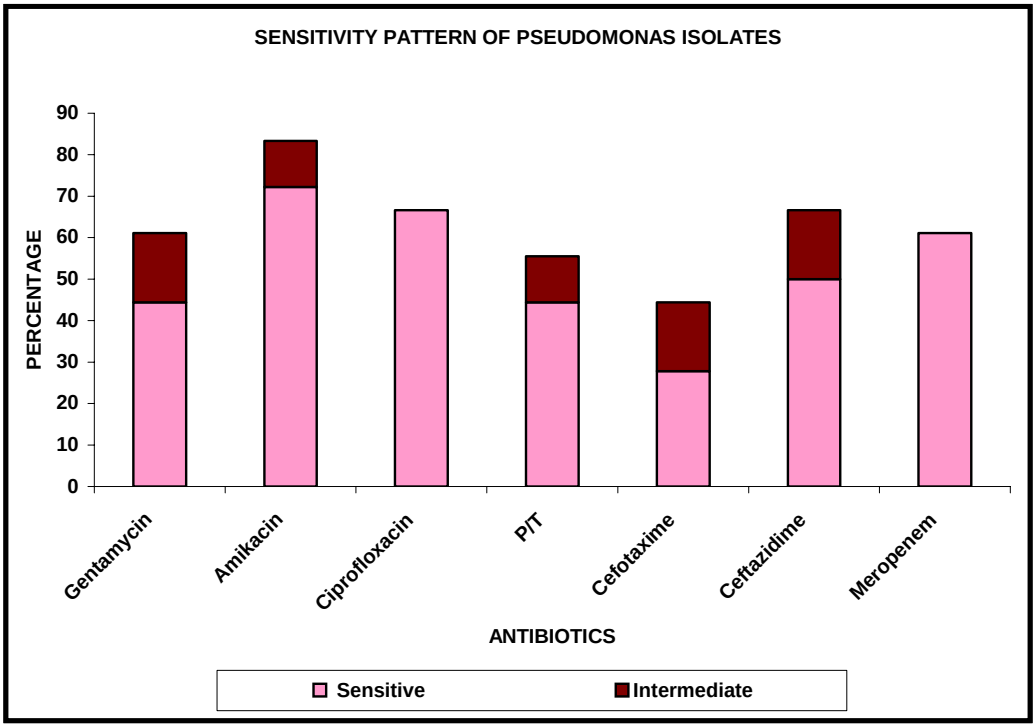


TABLE – 7

Total No.of MBL producers among various NFGNB (n = 22)

Organism	Total No. of Isolates	MBL Producers	Non -MBL producers
Pseudomonas	18	7 (38.88%)	11 (61.11%)
Acinetobacter	2	-	2
Others (Chryseobacterium & Shewanella)	2	-	2

Out of 18 Pseudomonas isolates, 7 were found to be MBL Producing organisms which accounted for 38.88%. No MBL producing isolates were seen among other NFs.

TABLE – 8

Total No. of MBL producers among Pseudomonas

Organism	Total No. of Isolates	MBL Producers	Non-MBL producers
P.aeruginosa	15	5	10
P.fluorescens	3	2	1
Total	18	7	11

Out of 7 MBL producing Pseudomonas, 5 were *P.aeruginosa* and remaining 2 were *P.fluorescens*.

DISTRIBUTION OF MBL PRODUCERS AMONG PSEUDOMONAS ISOLATES

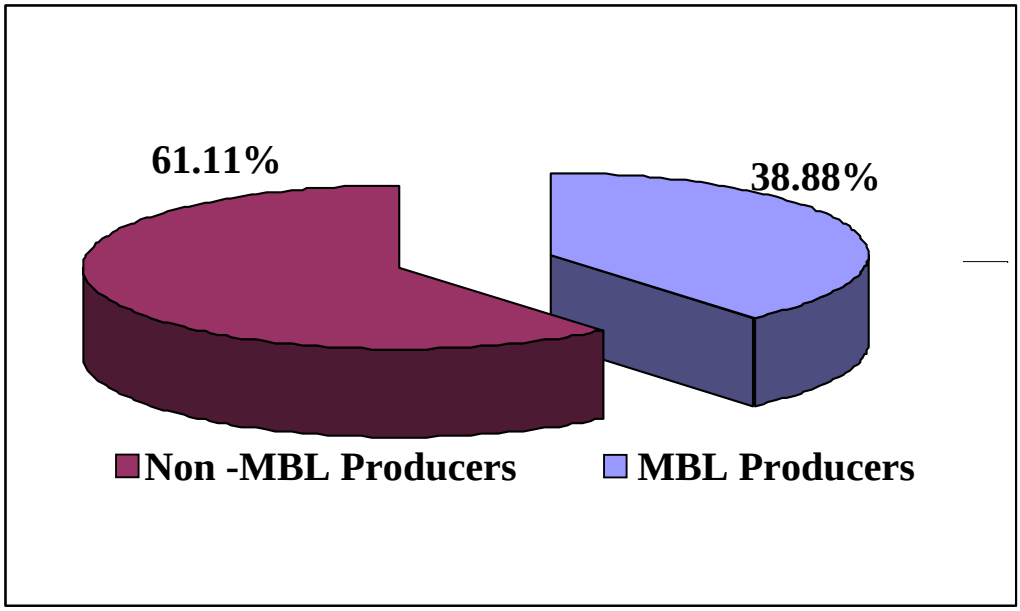


TABLE 9

Zone enhancement of MBL producing Pseudomonas

Organism	Total No.of MBL producers	Zone enhancement with both CaEDTA / MeEDTA	Zone enhancement with CaEDTA only	Zone enhancement with MeEDTA only
P.aeruginosa	7	5	1	1

CaEDTA – Ceftazidime EDTA disc, MeEDTA – Meropenem EDTA disc

Screening for MBL production was done by Double-Disc synergy test using EDTA impregnated Meropenem and Ceftazidime disc. Of the 7 MBL producers, 5 isolates showed zone enhancement with both CaEDTA and MeEDTA. 1 isolate showed zone enhancement only with CaEDTA. The remaining 1 isolate showed zone enhancement with only MeEDTA.

TABLE 10

Percentage of isolates sensitive to different betalactam antibiotics among MBL producers and non-MBL producers

	Cefotaxime	Ceftazidime	P/T
MBL Producers (n = 7)	28.57 %	57.14%	42.85%
Non-MBL producers (n = 11)	54.54%	72.72%	63.63%

TABLE – 11

Resistance of P.aeruginosa strains to meropenem, Ceftazidime (or) Ciprofloxacin according to previous therapy with meropenem / third generation Cephalosporins / Ciprofloxacin (or) any fluoroquinolone

Strain resistant	Previous therapy with meropenem		Previous therapy with 3rd generation Cephalosporins		Previous therapy with fluoroquinolone	
	No	Yes	No	Yes	No	Yes
To meropenem	3	4	4	3	2	5
To Ceftazidime	4	2	3	3	2	4
To Ciprofloxacin	5	1	2	4	1	5

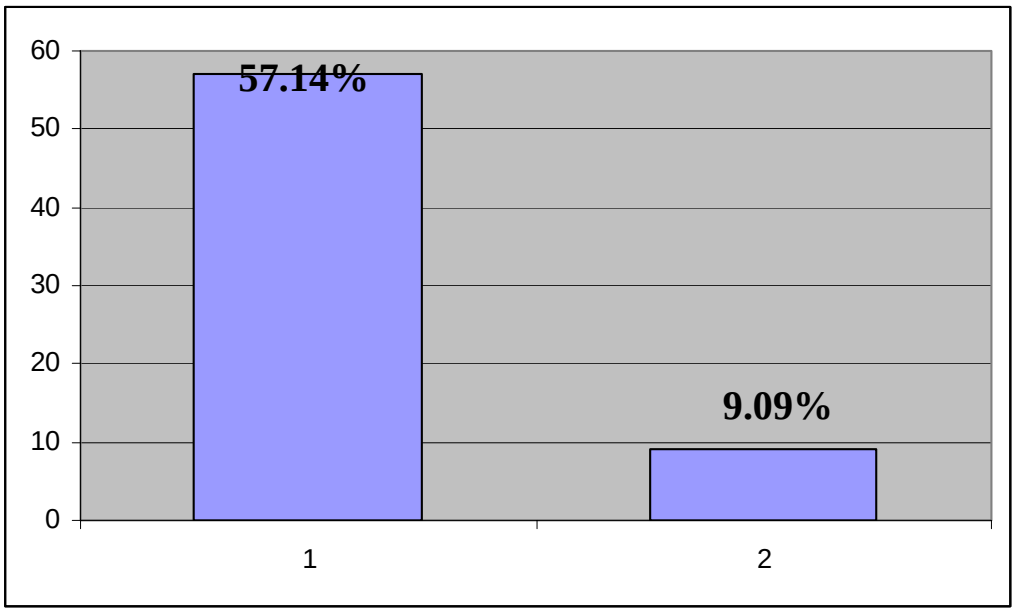
TABLE – 12

Mortality Rates among hospitalized, critically ill patients with infections caused by MBL producing P.aeruginosa

P.aeruginosa	Total No.Isolates	Mortality (%) of Patients
MBL producers	7	4 (57.14%)
Non-MBL producers	11	1 (9.09%)

By Chi-square test, the mortality rate among MBL Producers is statistically significant when compared to Non-MBL producers. (P = 0.03)

MORTALITY RATES AMONG MBL PRODUCERS



1. Mortality among MBL Producers
2. Mortality among Non-MBL Producers

Discussion

DISCUSSION

In the present study, 100 patients admitted to ICU and developing symptoms suggestive of infections were investigated. Hospital-acquired infections pose a challenge to the ICU patients and threatens to undo the therapeutic advantages of life saving interventions undertaken by clinicians.[9]

The present study obtained an isolation rate of 16.3% of non-fermenters among the total samples and Pseudomonas sp accounted for 13.3% of the infected patients which was observed in the studies of Richard MJ et al which showed an isolation rate of 13.2% to 44%.[45]

Among the NFs isolated from clinical specimens, 81.8% were identified as Pseudomonas species. Study conducted by Yasodhara and Shyamala obtained an isolation rate of 76%.[58]

In the present study an isolation rate of 68.18% of *P.aeruginosa* was obtained among NFs. The study of Kaushal et al showed an isolation rate of 88.8% of *P.aeruginosa*. [36] The study conducted by Yasodhara and Shyamala obtained an isolation rate of 57% of *P.aeruginosa*. [58] Study conducted by Gladstone, Rajendaran and KN.Brahmadathan shows an isolation rate of 42.8% of *P.aeruginosa*. [43] The above studies are almost in correlation with the present study.

The present study showed an isolation rate of 13.63% of *P.fluorescens*. The other NFs isolated were *A.baumannii, A.lwofii, Chryseobacterium* and *Shewanella* species with an isolation rate of 4.54%.

The study conducted by Veenu et al showed an isolation rate of 3.8% of *P.fluorescens* and 0.63% of *A.baumannii* and *A.lwofii*.[57] Another study

conducted by Yashodhara and Shyamala showed an isolation rate of 8% of *P.fluorescens* and 13% of *A.baumannii* and 2% of *A.lwofii*. [58]

Maximum isolation rate of NFs were from endotracheal aspirate (40%), indicating that the commonest site of Pseudomonas infection in ICU patients was the respiratory tract. Second common specimen with maximum isolation was pus (18.18%).

Next commonest sample with maximum isolation were urine (16.13%), blood (12%) and sputum (12%). Study conducted by Mishra et al found that most of the strains were from pus and blood. [58]

In our study sensitivity rate of Pseudomonas isolates to gentamycin was 61%, Amikacin - 83.33%, Ciprofloxacin – 66.6%, Piperacillin / Tazobactam – 55.6%, Cefotaxime – 54.4%, Ceftazidime – 66.7% and Meropenem – 61.1%.

Amikacin (83.33%) was the highly effective anti-pseudomonal agent. This correlates with the study conducted by Nagoba et al which also showed amikacin as the most effective anti-pseudomonal agent. [6] The results of aminoglycoside sensitivity indicate that reistance to gentamycin is increasing. This correlates with the finding in the study conducted by Zheng YN et al. [60]

Out of 15 isolates of *P.aeruginosa*, 80% were found to be sensitive to amikacin, 66.6% to gentamycin, ciprofloxacin and meropenam, 60% to piperacillin-tazobactam, 73.3% to ceftazidime and 53.3% to cefotaxime.

The study conducted by Nagoba et al which shows 97.7% of P.aeruginosa strains was sensitive to amikacin, 80% to ciprofloxacin and 53.3% to gentamycin. [6]

Sensitivity of *P.aeruginosa* to meropenem was 66.6% in our study. The study conducted by Sarkar et al showed a sensitivity rate of 59.09%[25] and another study shows the sensitivity rate of 88% to Carbapenems. [43] Other NFs isolated in this study was sensitive to most of the antibiotics used like gentamycin, Amikacin, Ciprofloxacin, Ceftzadime and meropenem.

The pseudomonas isolates of all species showed alarming levels of resistance to even the most recent antibiotics like third generation cephalosporins, anti-pseudomonal penicillins, aminoglycosides and fluoroquinolones. Resistance of such a high order could be because of unrestricted use of antibiotics.

55.5% of pseudomonas isolates were found to exhibit multidrug resistance pattern- isolates resistant to two or more drugs. The study conducted by Veenu et al showed a multidrug resistant pattern with 65.3% of isolates.[57] Similar view regarding the correlation between infection with *P.aeruginosa* and previous antibiotic use has also been reported by Bowton.

This study documents a carbapenem resistance of 33.3% among *P.aeruginosa* and 38.9% of carbapenem resistance among all pseudomonas isolates. This is in contrast to the study conducted by Gladstone et al which documents 12.2% resistance to carbapenem.[43] Study conducted by Navaneeth et al reported a prevalence of 12% carbapenem resistance. [39]

In view of the increasing reports of MBL production in pseudomonas, the isolates obtained in the present study were tested for the production of the same.

Of the 18 isolates of pseudomonas, 7 showed resistance to meropenem by the double disc synergy method. All the 7 isolates exhibited a

significant zone size enhancement with the EDTA impregnated discs when compared with the plain antibiotic discs, which indicated MBL production by these isolates.

5 out of 7 isolates had zone size enhancement with both ceftazidime EDTA and meropenem EDTA discs. The zone size enhancement was 5-28mm for ceftazidime EDTA and 7-27mm for meropenemEDTA discs.

One isolate exhibited a zone size increase only with ceftazidimeEDTA disc and one isolate only with meropenem EDTA disc. The remaining 11 isolates were susceptible to meropenem and showed a zone size increase of only 0-5mm with EDTA impregnated antibiotic discs.

In this study 38.9% of Pseudomonas isolates were found to be MBL producers. This is high when compared to 12% of MBL-mediated meropenem resistance in study conducted by Navaneeth et al.[39] Another study conducted by Hemalatha et al showed 16% MBL production among pseudomonas. [26] Study conducted by Sarkar et al. showed 54.5% of Pseudomonas isolates were MBL producers.

Mortality rates among MBL Producers were 57.14% when compared to 9.09% among Non-MBL producing P.aeruginosa. MBL production is a significant problem in hospital isolates of pseudomonas. With increasing use of carbapenems, the problem of MBL production is also increasing. The development of simple screening tests, designed to detect MBL production will be a crucial step towards large scale monitoring of these emerging resistant determinants. The use of EDTA impregnated antibiotic disc is a simple, easy to perform and cost effective test which can be conveniently used to screen carbapenem resistance. [26]

Summary

SUMMARY

- The study was done at Department of Microbiology during June 2006 to May 2007. In the Study 135 samples from 100 cases admitted to ICU developing symptoms suggestive of infections were collected and categorised.

- By following routine standard isolation techniques, a total of 22 NFs (16.3%) were isolated from 135 clinical specimens.

- *P.aeruginosa* was the commonest NF isolated, irrespective of clinical samples.

- Though NFs are considered as opportunistic pathogens, it was observed in the present study, *P.fluorescens, A.baumannii, A.lwofii,* Chryseobacterium and Shewanella also could establish clinical infection like *P.aeruginosa* in immunocompromised patients.

- Antibiotic susceptibility pattern was observed for these organism and it showed multidrug resistance pattern with majority of isolates being resistant to two (or) more drugs.

- Screening for the production of MBL among Pseudomonas in this study revealed 38.9% were MBL producers.

- The clinical outcome included in the study showed high rate of mortality (57.14%) among MBL producers than non-MBL producers and it was found to be statistically significant.

Conclusion

CONCLUSION

- Infections due to NFs pose a challenge to the ICU patients.
- NFGNB, normally a saprophyte, cause serious infections in immunocompromised and hospitalized patients especially those admitted to ICU.
- Of the various agents of NFs, Pseudomonas was the commonest organism to be isolated.
- Multidrug resistant Pseudomonas were observed. Amikacin was the highly effective antibiotic against most of the isolates.
- Increased incidence of MBL producing Pseudomonas had been observed.
- Mortality rate were high among MBL producers than non-producers.

Appendix

APPENDIX

MacConkey agar

This is a useful medium for the cultivation of enterobacteriaceae. It contains a bile salt to inhibit non-intestinal bacteria and lactose with neutral red to distinguish the lactose-fermenting coliforms from the non lactose fermenting salmonella and shigella groups. The concentration of sodium taurocholate may be reduced to suit less tolerant organisms. The omission of sodium chloride from the medium prevents the spreading of proteus colonies.

Peptone	20g
Sodium taurocholate, commercial	5 g
Water	1 litre
Agar	20 g
Neutral red solution, 2% in 50% ethanoal	3.5 ml
Lactose, 10% aqueous solution	100 ml

Dissolve the peptone and taurocholate (bile salt) in the water by heating. Add the agar and dissolve it in the steamer or autoclave. If necessary, clear by filtration. Adjust the pH to 7.5. Add the lactose and the neutral red, which should be well shaken before use, and mix. Heat in the autoclave with 'free steam' (c. 100^{o} C) for 1 hr., then at 115^{o} C for 15 min. Pour plates.

Nutrient agar

	Gm/L
Peptic digest of animal tissue	5.00
Beef extract	1.50
Yeast extract	5.00
Agar	15.00

Dissolve the contents in water and mix by heating. Autoclave at 121^{o} C for 15 minutes. Adjust pH to 7.4. Pour 20-25 ml of 9 cm dia. Petridishes to give 4 mm thickness.

Blood agar

Sterile sheep blood	50 ml
Peptone	10 g
Beef extract	3 g
Sodium chloride	5 g
Distilled water	1000 ml

Autoclave the nutrient agar base at 121^{o} C for 15 minutes and blood with sterile precautions and distribute in Petri dishes.

Muller Hinton agar

Beef infusion	300 ml
Casein Hydrolysate	17 gm
Starch	1.5 gm
Agar	10 gm
Distilled water	1000 ml

Emulsify the starch in a small amount of cold water, pour into the beef infusion and add the casein-Hydrolysate and the agar. Make up the volume to 1000 ml (1 litre) with distilled water. Dissolve the constituents by heating gently at 100^{o}C with agitation.

Adjust the pH to 7.4. Dispense in screw-capped bottles and sterilize by autoclaving at 121^{o} C for 20 minutes. 20 to 25 ml of it is poured into petridishes of 9 cm diameter to give a thickness of 4 mm.

McFarland's Turbidity Standard for inoculum preparation

A Barium sulphate 0.5 McFarland standard was prepared as follows

1. A 0.5 ml of 0.048 mol/L of Barium chloride was added to 99.5 ml of 0.18 mol/L of H_2SO_4 with constant stirring to maintain a suspension.

2. Correct density of the turbidity standard was verified by using a spectrophotometer. The absorbance of 625nm should be 0.08 to 0.10 for the 0.5 McFarland standards.

3. The Barium sulphate suspension was transferred in 4-6 ml to a screw capped tube of the same size as those used in growing or diluting the bacterial inoculum.

4. These tubes were tightly sealed and stored in the dark at room temperature.

5. The Barium sulphate turbidity standard was vigorously agitated before each use and inspected for a uniform turbid appearance.

PROFORMA

NAME AGE SEX

COMPLAINTS

PAST HISTORY

H/O MECHANICAL VENTILATION

H/O ANY SURGERY

DURATION OF STAY IN HOSPITAL

TREATMENT HISTORY (ANY ANTIBIOTICS GIVEN)

CLINICAL FINDINGS

DIAGNOSIS

INVESTIGATIONS

RESPONSE TO TREATMENT

MASTER CHART

Sl.No	IP No	Age/Sex	Sample	Organism	G	AK	Cp	P/T	Ce	Cz	Me	MBL Producers
1	023503	23/F	Blood	*P.aeruginosa *P.aeruginosa	R R	S S	R R	R R	R R	R R	R R	Positive
2	071425	54/M	Blood	*P.aeruginosa *P.aeruginosa	R R	R R	R R	R R	R R	R R	R R	Positive
3	028627	33/M	Blood	*A.calcoaceticus *A.calcoaceticus	S R	S S	S S	S R	R R	S S	S S	Negative
4	024414	16/M	Pus	*P.aeruginosa *P.aeruginosa	S S	S S	S S	S S	R R	S S	S S	Negative
5	023081	52/M	Pus	*P.aeruginosa *P.aeruginosa	S R	S S	S S	S S	R R	S S	S S	Negative
6	071665	17/F	Pus	*P.aeruginosa *P.aeruginosa	R R	R R	R R	R R	R R	R R	R R	Positive
7	071404	23/M	Pus	*P.fluorescens *P.fluorescens	S S	S S	S S	R R	R R	S S	S S	Negative
8	023500	25/M	Pus	*C.meningosepticum *C.meningosepticum	S S	S S	S S	S S	S S	S S	S S	Negative
9	023444	75/F	Pus	*S.Putrefaciens *S.Putrefaciens	R R	R R	S S	R R	R F	S S	S S	Negative
10	071005	16/M	Sputum	*P.aeruginosa *P.aeruginosa	S S	S S	S S	S S	S S	S S	S S	Negative
11	023810	29/M	Sputum	*P.aeruginosa *P.aeruginosa	S R	S S	S S	R R	R R	S S	S S	Negative
12	023719	17/F	Sputum	*P.aeruginosa *P.aeruginosa	S S	S S	S S	S S	S S	S S	S S	Negative
13	018910	36/M	Urine	*P.aeruginosa *P.aeruginosa	S S	S S	S S	S S	S S	S S	S S	Negative
14	021110	17/F	Urine	*P.aeruginosa *P.aeruginosa	S S	S S	S S	S S	R S	S S	S S	Negative
15	024211	42/M	Urine	*P.aeruginosa *P.aeruginosa	R R	S S	R R	R R	R R	R R	R R	Positive
16	071200	36/F	Urine	*P.aeruginosa *P.aeruginosa	S R	S S	S S	R R	S R	S S	S S	Negative
17	025112	43/F	Urine	*P.fluorescens *P.fluorescens	R R	R R	R R	R R	R R	R R	R R	Positive
18	071311	17/M	Endoracheal Aspirate	*P.aeruginosa *P.aeruginosa	S S	S S	S S	S S	S S	S S	S S	Negative
19	023175	50/F	E.aspirate	*P.aeruginosa *P.aeruginosa	R R	S S	R R	R R	R R	R R	R R	Positive
20	071325	25/F	E.aspirate	*P.aeruginosa *P.aeruginosa	S S	S S	S S	S S	S S	S S	S S	Negative
21	024127	52/M	E.aspirate	*P.fluorescens *P.fluorescens	R R	S S	S S	S R	R R	S S	R R	Positive
22	023317	40/M	BAL	*A.lwofii *A.lwofii	S S	S S	S S	S S	S S	S S	S S	Negative

G - Gentamycin, AK - Amikacin, Cp - Ciprofloxacin, P/T – Piperacillin – Tazobactam,
Ce – Cefotaxime, Cz – Ceftazidime, Me – Meropenem,
* - Organisms obtained by repeat Isolation
MBL – Metallo Beta Lactamases

Sl.No	IP.No	Age / Sex	Sample	Organism isolated
23	023085	15/F	Blood	K.pnemoniae
24	023446	75/M	Blood	K.oxytoca
25	071220	17/F	Blood	K.oxytoca
26	071301	30/F	Blood	K.oxytoca
27	071329	15/M	Blood	K.oxytoca
28	024098	20/M	Blood	K.pnemoniae
29	071453	22/M	Blood	K.pnemoniae
30	021307	23/F	Blood	K.pnemoniae
31	071458	17/F	Blood	P.aeruginosa
32	071625	25/M	Blood	P.aeruginosa
33	025098	29/M	Blood	E.coli
34	071034	37/F	Blood	E.coli
35	070883	31/M	Blood	Micrococcus
36	071663	17/F	Blood	CONS
37	025309	18/M	Blood	CONS
38	024098	20/M	Blood	Micrococcus
39	071427	49/M	Blood	NG
40	071426	37/M	Blood	NG
41	070982	42/M	Blood	NG
42	023496	75/M	Blood	NG
43	023028	47/M	Blood	CONS
44	023812	28/M	Blood	CONS
45	023446	75/M	Pus	P.mirabilis
46	023452	30/F	Pus	P.mirabilis
47	024215	73/M	Pus	P.mirabilis
48	023085	15/F	Pus	P.vulgaris
49	02342	28/M	Pus	P.vulgaris
50	024213	73/M	Pus	P.vulgaris
51	071220	17/F	Pus	P.vulgaris
52	071301	30/F	Pus	P.vulgaris
53	071329	15/F	Pus	P.aeruginosa
54	024217	17/F	Pus	K.oxytoca
55	071425	37/M	Pus	K.oxytoca
56	071427	49/M	Pus	C.freundii
57	071426	37/M	Pus	K.pneumonia
58	071317	31/M	Pus	K.oxytoca
59	071327	40/F	Pus	K.oxytoca
60	070809	36/F	Pus	K.pneumonia
61	023690	40/M	Pus	S.aureus
62	070376	17/F	Pus	S.aureus

Sl.No	IP.No	Age / Sex	Sample	Organism isolated
63	070936	51/F	Pus	CONS
64	024431	37/M	Pus	Diphtheroids
65	071493	25/M	Pus	CONS
66	070883	52/F	Pus	CONS
67	070310	52/F	Pus	Diphtheroids
68	071034	37/F	Pus	P.aeruginosa
69	04625	25/M	Pus	P.aeruginosa
70	070710	44/F	Pus	Pneumococcus
71	271506	78/M	Pus	Pneumococcus
72	023812	28/M	Sputum	K.oxytoca
73	023496	75/M	Sputum	K.oxytoca
74	023028	47/M	Sputum	K.pneumoniae
75	02342	28/M	Sputum	K.pneumoniae
76	028627	33/M	Sputum	K.pneumoniae
77	023503	22/M	Sputum	Candida sp
78	023747	16/F	Sputum	P.vulgaris
79	021332	30/F	Sputum	P.mirabilis
80	018912	38/M	Sputum	P.mirabilis
81	023910	52/F	Sputum	P.mirabilis
82	021124	74/M	Sputum	CONS
83	024098	20/M	Sputum	CONS
84	023446	75/M	Sputum	CONS
85	023085	15/F	Sputum	CONS
86	024213	73/M	Sputum	CONS
87	024446	40/M	Sputum	Diphtheroids
88	071223	19/F	Sputum	Diphtheroids
89	025101	30/F	Sputum	S.aureus
90	024110	17/M	Sputum	S.aureus
91	023012	29/M	Sputum	S.aureus
92	070661	30/M	Sputum	S.aureus
93	022123	45/M	Sputum	NG
94	071302	32/M	Urine	E.coli
95	071450	40/F	Urine	E.coli
96	024190	19/F	Urine	E.coli
97	023080	51/M	Urine	E.coli
98	023075	50/F	Urine	E.coli
99	070300	23/F	Urine	E.coli
100	071325	25/F	Urine	E.coli
101	023494	55/F	Urine	E.coli
102	023259	23/M	Urine	K.oxytoca
103	024429	36/M	Urine	K.oxytoca

Sl.No	IP.No	Age / Sex	Sample	Organism isolated
104	025101	40/M	Urine	K.oxytoca
105	070911	50/F	Urine	K.pneumoniae
106	024309	36/M	Urine	K.pneumoniae
107	071401	42/F	Urine	K.pneumoniae
108	071601	45/M	Urine	K.pneumoniae
109	070880	52/M	Urine	P.aeruginosa
110	025317	50/M	Urine	P.aeruginosa
111	024127	52/M	Urine	P.vulgaris
112	072011	23/M	Urine	P.vulgaris
113	021122	41/F	Urine	Enterococci
114	024185	42/M	Urine	Enterococci
115	070111	51/F	Urine	Enterococci
116	070890	21/F	Urine	NG
117	024198	23/F	Urine	NG
118	028520	25/M	Urine	NG
119	025199	26/F	Urine	NG
120	023081	17/F	CSF	NG
121	071663	17/F	CSF	NG
122	070982	42/M	CSF	NG
123	071426	37/M	CSF	NG
124	023912	71/F	CSF	K.pneumoniae
125	021330	21/F	Endotracheal swab	K.pneumoniae
126	071427	49/M	Endotracheal swab	K.pneumoniae
127	070376	17/F	Endotracheal swab	K.oxytoca
128	071223	19/F	Endotracheal swab	Diphtheroids
129	022001	27/M	Endotracheal swab	CONS
130	025211	32/F	Endotracheal swab	CONS
131	023317	40/M	Pleural fluid	K.pneumoniae
132	025121	40/M	Pleural fluid	K.pneumoniae
133	018802	32/M	Pleural fluid	K.pneumoniae
134	021998	23/F	Pleural fluid	NG
135	072221	17/F	Pleural fluid	NG

KEY TO MASTER CHART

M	-	Male
F	-	Female
CSF	-	Cerebrospinal Fluid
K.pneumoniae	-	Klebsiella pneumoniae
K.oxytoca	-	Klebsiella oxytoca
E.coli	-	Escherichia coli
CONS	-	Coagulase Negative Staphylococcus
P.mirabilis	-	Proteus mirabilis
P.vulgaris	-	Proteus vulgaris
NG	-	No growth

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